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Dated 4 January 2005

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# Request for grant of a patent

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1. Your reference — P34234-/CMU/MCM

26NOV03 E855185-1 D02884
P01/7700 0.00-0327493.3

2. Patent application numbe *(The Patent Office will fill in th* — 0327493.3

26 NOV 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

The Queen's University of Belfast
University Road
Belfast
BT7 1NN

Patents ADP number *(if you know it)* 8896590006

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — "Treatment Medicament"

5. Name of your agent *(if you have one)*

Murgitroyd & Company

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Scotland House
165-169 Scotland Street
Glasgow
G5 8PL

Patents ADP number *(if you know it)* 1198013

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

| Country | Priority application number *(if you know it)* | Date of filing *(day / month / year)* |
|---|---|---|

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

| Number of earlier application | Date of filing *(day / month / year)* |
|---|---|

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

*a) any applicant named in part 3 is not an inventor, or*
*b) there is an inventor who is not named as an applicant, or*
*c) any named applicant is a corporate body.*
*See note (d))*

Yes

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9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

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| Continuation sheets of this form | |
| Description | 59 |
| Claim(s) | 5 |
| Abstract | - |
| Drawing(s) | 20 |

10. If you are also filing any of the following, state how many against each item.

| | |
|---|---|
| Priority documents | |
| Translations of priority documents | |
| Statement of inventorship and right to grant of a patent *(Patents Form 7/77)* | |
| Request for preliminary examination and search *(Patents Form 9/77)* | |
| Request for substantive examination *(Patents Form 10/77)* | |
| Any other documents *(please specify)* | |

11. I/We request the grant of a patent on the basis of this application.

Signature Murgitroyd & Company — Date 26 November 2003

12. Name and daytime telephone number of person to contact in the United Kingdom: MALCOLM MAIN — 0141 307 8400

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# "Treatment Medicament"

## Field of the Invention

This application relates to a medicament and its use in methods of treatment. In particular, it relates to the treatment of cancer with a death receptor ligand, e.g. a FAS (CD95 or TNF receptor 2) receptor ligand, and a chemotherapeutic agent.

## Background to the Invention

Breast, oesophageal, colorectal, all forms of GI cancer and head and neck cancers are highly malignant with overall 5-year survival rates of less than 50%. The clinical outcome of these patients is predetermined by the presence of widely disseminated tumour cells termed micrometastases with potential for metastatic growth, prior to clinical presentation. Approximately 50% of oesophageal cancer patients are selected for surgical therapy

with 30% 5-year survival for this patient sub-group. Randomised clinical trials of neoadjuvant 5FU-based chemotherapy combined with fractionated radiotherapy have demonstrated improvements in survival of 10-20%, although the overall 5-year outcome for the treated groups remains at 30-35%. Those patients who demonstrate complete pathological response in their primary tumours as a result of neoadjuvant treatment have a five-year survival of 80%. Conversely, those patients who do not respond to 5FU-based chemotherapy are denied the opportunity for earlier treatment by surgery or a different neoadjuvant chemotherapeutic based regimen. Thus, there is an urgent need for improved therapeutic strategies.

5-FU[4] is widely used in the treatment of a range of cancers including colorectal, breast and cancers of the aerodigestive tract. The mechanism of cytotoxicity of 5-FU has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS) (1). TS catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5,10-methylene tetrahydrofolate ($CH_2THF$) as the methyl donor. This reaction provides the sole intracellular source of thymidylate, which is essential for DNA synthesis and repair. The 5-FU metabolite fluorodeoxyuridine monophosphate (FdUMP) forms a stable complex with TS and $CH_2THF$ resulting in enzyme inhibition (1). Recently, more specific

folate-based inhibitors of TS have been developed such as RTX and MTA, which form a stable complex with TS and dUMP that inhibits binding of $CH_2THF$ to the enzyme (2, 3). TS inhibition causes nucleotide pool imbalances that result in S phase cell cycle arrest and apoptosis (4-6).

## Summary of the Invention

As described herein, the present inventors have surprisingly shown that by combining treatment using a death receptor ligand, such as an anti FAS antibody, with a chemotherapeutic agent such as 5-FU or an antifolate drug, such as ralitrexed (RTX) or pemetrexed (MTA, Alimta), a synergistic effect is achieved in the killing of cancer cells.

Accordingly, in a first aspect, the present invention provides a method of killing cancer cells comprising administration of a therapeutically effective amount of a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent.

In a second aspect, the present invention provides a method of treating cancer comprising administration of a therapeutically effective amount of a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding

member and (b) a chemotherapeutic agent to a mammal in need thereof.

The specific binding member and the chemotherapeutic agent may be administered simultaneously, sequentially or simultaneously. In preferred embodiments of the invention, the chemotherapeutic agent is administered prior to the specific binding member.

In a third aspect, there is provided the use of (a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent in the preparation of a medicament for treating cancer.

In a fourth aspect, there is provided a product comprising a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent as a combined preparation for the simultaneous, separate or sequential use in the treatment of cancer.

According to a fifth aspect, there is provided a pharmaceutical composition for the treatment of cancer, wherein the composition comprises a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent and (c) a pharmaceutically acceptable excipient, diluent or

carrier.

In a sixth aspect, there is provided a kit for the treatment of cancer, said kit comprising a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent and (c) instructions for the administration of (a) and (b) separately, sequentially or simultaneously.

The invention may be used to treat any cancer. In preferred embodiments of the invention, the cancer is one or more of colorectal, breast , ovarian, cervical, gastric, lung, liver, skin and myeloid (e.g. bone marrow) cancer.

In preferred embodiments of the invention, the binding member is an antibody or a fragment thereof. In particularly preferred embodiments, the binding member is the FAS antibody CH11 (Yonehara, S., Ishii, A. and Yonehara, M. (1989) J. Exp. Med. 169, 1747-1756) (available commercially e.g. from Upstate Biotechnology, Lake Placid, NY).

The binding member may bind to any death receptor. Death receptors include, Fas, TNFR, DR-3, DR-4 and DR-5. In preferred embodiments of the invention, the death receptor is FAS.

In preferred embodiments, the binding member comprises at least one human constant region.

Any suitable chemotherapeutic agent may be used in the present invention. In preferred embodiments, the agent is doxorubicin, oxaliplatin, taxol, tomudex (TDX), 5-Fluorouracil (5-FU), Irinotecan (CPT11) or an antifolate e.g. MTA or RTX. In one preferred embodiment, the agent is tomudex, 5-Fluorouracil, an antifolate (for example RTX or MTA), or a combination thereof. In a particularly preferred embodiment, the agent is 5-FU or an antifolate. In another preferred embodiment, the agent is an antifolate. In a particularly preferred embodiment the agent is MTA.

The invention also provides a method of treating tumour cells, the method including the steps of administering a compound capable of triggering or binding a death receptor, e.g. a binding member and administering a chemotherapeutic agent.

The concentrations of binding members and chemotherapeutic agents used are preferably sufficient to provide a synergistic effect. Synergism is preferably defined as an RI of greater than unity using the method of Kern as modified by Romaneli (13, 14). The RI may be calculated as the ratio of expected cell survival ($S_{exp}$, defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) to the observed cell survival ($S_{obs}$) for the combination of A and B ($RI=S_{exp}/S_{obs}$). Synergism may then be defined as an RI of greater than unity.

In preferred embodiments of the invention, said specific binding member and chemotherapeutic agent are provided in concentrations sufficient to produce an RI of greater than 1.5, more preferably greater than 2.0, most preferably greater than 2.25.

The combined medicament thus preferably produces a synergistic effect when used to treat tumour cells.

A seventh aspect of the present invention therefore provides a medicament for use in treating tumour cells, the medicament comprising at least one antibody directed at FAS receptor and at least one cancer chemotherapeutic agent.

The invention also provides in a eighth aspect a method of treating tumour cells, the method including the steps of administering a compound capable of triggering or binding a death receptor and administering simultaneously, sequentially or separately a chemotherapeutic agent.

In an ninth aspect, the invention provides the use of an antibody directed at FAS receptor in combination with a cancer chemotherapeutic agent in the preparation of a medicament for treatment of tumour cells.

In a particular aspect, the application relates to the use of an antibody or a fas ligand directed at a death receptor e.g. the FAS receptor (CD95/TNF receptor 2) to synergise with cancer

chemotherapeutic agents, e.g. 5-FU or an antifolate, for example RTX or MTA, to enhance therapy and enhance the removal or regression of tumour cells.

This application is relevant for, but is not limited to, breast cancer, oesophageal cancer, colorectal cancer, all forms of GI cancer and head and neck cancers and may also be used to target other cells via death receptors.

Preferred features of each aspect of the invention are as for each of the other aspects mutatis mutandis.

**Detailed Description**

**Binding members**

In the context of the present invention, a "binding member" is a molecule which has binding specificity for another molecule, in particular a receptor, in particular a death receptor. The binding member may be a member of a pair of specific binding members. The members of a binding pair may be naturally derived or wholly or partially synthetically produced. One member of the pair of molecules may have an area on its surface, which may be a protrusion or a cavity, which specifically binds to and is therefore complementary to a particular spatial and polar organisation of the other member of the pair of molecules. Thus, the members of the pair have the property of binding specifically to

each other. Examples of types of binding pairs are antigen-antibody, biotin-avidin, hormone-hormone receptor, receptor-ligand, enzyme-substrate. A binding member of the invention and for use in the invention may be any moiety, for example an antibody or ligand, which can bind to a death receptor.

**Antibodies**

An "antibody" is an immunoglobulin, whether natural or partly or wholly synthetically produced. The term also covers any polypeptide, protein or peptide having a binding domain which is, or is homologous to, an antibody binding domain. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of antibodies are the immunoglobulin isotypes and their isotypic subclasses and fragments which comprise an antigen binding domain such as Fab, scFv, Fv, dAb, Fd; and diabodies.

The binding member of the invention may be an antibody such as a monoclonal or polyclonal antibody, or a fragment thereof. The constant region of the antibody may be of any class including, but not limited to, human classes IgG, IgA, IgM, IgD and IgE. The antibody may belong to any sub class e.g. IgG1, IgG2, IgG3 and IgG4. IgG1 is preferred.

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any binding member or substance having a binding

domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or wholly or partially synthetic. Chimeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of such binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341:544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al., Science 242:423-426 (1988); Huston et al., PNAS USA 85:5879-5883 (1988)); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed

by gene fusion (WO94/13804; P. Hollinger et al., Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993)).

A fragment of an antibody or of a polypeptide for use in the present invention generally means a stretch of amino acid residues of at least 5 to 7 contiguous amino acids, often at least about 7 to 9 contiguous amino acids, typically at least about 9 to 13 contiguous amino acids, more preferably at least about 20 to 30 or more contiguous amino acids and most preferably at least about 30 to 40 or more consecutive amino acids.

A "derivative" of such an antibody or polypeptide, or of a fragment antibody means an antibody or polypeptide modified by varying the amino acid sequence of the protein, e.g. by manipulation of the nucleic acid encoding the protein or by altering the protein itself. Such derivatives of the natural amino acid sequence may involve insertion, addition, deletion and/or substitution of one or more amino acids, preferably while providing a peptide having death receptor, e.g. FAS neutralisation and/or binding activity. Preferably such derivatives involve the insertion, addition, deletion and/or substitution of 25 or fewer amino acids, more preferably of 15 or fewer, even more preferably of 10 or fewer, more preferably still of 4 or fewer and most preferably of 1 or 2 amino acids only.

The term "antibody" includes antibodies which have been "humanised". Methods for making humanised

antibodies are known in the art. Methods are described, for example, in Winter, U.S. Patent No. 5,225,539. A humanised antibody may be a modified antibody having the hypervariable region of a monoclonal antibody and the constant region of a human antibody. Thus the binding member may comprise a human constant region.

The variable region other than the hypervariable region may also be derived from the variable region of a human antibody and/or may also be derived from a monoclonal antibody. In such case, the entire variable region may be derived from murine monoclonal antibody and the antibody is said to be chimerised. Methods for making chimerised antibodies are known in the art. Such methods include, for example, those described in U.S. patents by Boss (Celltech) and by Cabilly (Genentech). See U.S. Patent Nos. 4,816,397 and 4,816,567, respectively.

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementary determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma or

other cell producing an antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

A typical antibody for use in the present invention is a humanised equivalent of CH11 or any chimerised equivalent of an antibody that can bind to the FAS receptor and any alternative antibodies directed at the FAS receptor that have been chimerised and can be use in the treatment of humans. Furthermore, the typical antibody is any antibody that can cross-react with the extracellular portion of the FAS receptor and either bind with high affinity to the FAS receptor, be internalised with the FAS receptor or trigger signalling through the FAS receptor.

**Production of Binding Members**

The binding members for use in the present invention may be generated wholly or partly by chemical synthesis. The binding members can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they

may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

Another convenient way of producing a binding member suitable for use in the present invention is to express nucleic acid encoding it, by use of nucleic acid in an expression system. Thus the present invention further provides the use of (a) nucleic acid encoding a specific binding member which binds to a cell death receptor and (b) a chemotherapeutic agent in the preparation of a medicament for treating cancer.

Nucleic acid for use in accordance with the present invention may comprise DNA or RNA and may be wholly or partially synthetic. In a preferred aspect, nucleic acid for use in the invention codes for a binding member of the invention as defined above. The skilled person will be able to determine substitutions, deletions and/or additions to such nucleic acids which will still provide a binding member suitable for use in the present invention.

Nucleic acid sequences encoding a binding member for use with the present invention can be readily prepared by the skilled person using the information

and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning", A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al, Short Protocols in Molecular Biology, John Wiley and Sons, 1992), given the nucleic acid sequences and clones available. These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of such nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparing cDNA sequences. DNA encoding antibody fragments may be generated and used in any suitable way known to those of skill in the art, including by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Modifications to the sequences can be made, e.g. using site directed mutagenesis, to lead to the expression of modified peptide or to take account of codon preferences in the host cells used to express the nucleic acid.

The nucleic acid may be comprised as construct(s) in the form of a plasmid, vector, transcription or expression cassette which comprises at least one nucleic acid as described above. The construct may be comprised within a recombinant host cell which

comprises one or more constructs as above. Expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid. Following production by expression a specific binding member may be isolated and/or purified using any suitable technique, then used as appropriate.

Binding members-encoding nucleic acid molecules and vectors for use in accordance with the present invention may be provided isolated and/or purified, e.g. from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid or genes origin other than the sequence encoding a polypeptide with the required function.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, NSO mouse melanoma cells and many others. A common, preferred bacterial host is E. coli.

The expression of antibodies and antibody fragments in prokaryotic cells such as E. coli is well established in the art. For a review, see for example Plückthun, Bio/Technology 9:545-551 (1991). Expression in eukaryotic cells in culture is also

available to those skilled in the art as an option for production of a binding member, see for recent review, for example Reff, Curr. Opinion Biotech. 4:573-576 (1993); Trill et al., Curr. Opinion Biotech. 6:553-560 (1995).

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual: 2nd Edition, Cold Spring Harbor Laboratory Press (1989). Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Ausubel et al. eds., Short Protocols in Molecular Biology, 2nd Edition, John Wiley & Sons (1992).

The nucleic acid may be introduced into a host cell by any suitable means. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells,

suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for expression of the gene.

The nucleic acid may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

**Chemotherapeutic Agents**

As described above, the present invention is based on the surprising demonstration that combining treatment using a death receptor ligand such as the CH11 antibody with a chemotherapeutic agent results in a surprisingly enhanced synergistic therapeutic effect.

Any suitable chemotherapeutic agent or agents may be used in the present invention. For example, the agent for use in the invention may include but is not limited to: 5-Fluorouracil (5 FU), tomudex (TDX) antifolates, for example RTX or MTA, Doxorubicin, taxol, Leucovorin, Irinotecan, Mitomycin C, Oxaliplatin, Raltitrexed, Tamoxifen or Cisplatin.

In particularly preferred embodiments, the agent is 5-FU or an antifolate. More preferably, the agent is an antifolate. In one preferred embodiment, the agent is MTA.

**Treatment**

Treatment" includes any regime that can benefit a human or non-human animal. The treatment may be in respect of an existing condition or may be prophylactic (preventative treatment). Treatment may include curative, alleviation or prophylactic effects.

"Treatment of cancer" includes treatment of conditions caused by cancerous growth and includes the treatment of neoplastic growths or tumours. Examples of tumours that can be treated using the invention are, for instance, sarcomas, including osteogenic and soft tissue sarcomas, carcinomas, e.g., breast-, lung-, bladder-, thyroid-, prostate-, colon-, rectum-, pancreas-, stomach-, liver-, uterine-, cervical and ovarian carcinoma, lymphomas, including Hodgkin and non-Hodgkin lymphomas, neuroblastoma, melanoma, myeloma, Wilms tumor, and

leukemias, including acute lymphoblastic leukaemia and acute myeloblastic leukaemia, gliomas and retinoblastomas.

The compositions and methods of the invention may be particularly useful in the treatment of existing cancer and in the prevention of the recurrence of cancer after initial treatment or surgery.

**Administration**

Binding members and chemotherapeutic agents may be administered simultaneously, separately or sequentially.

Where administered separately or sequentially, they may be administered within any suitable time period e.g. within 1, 2, 3, 6, 12, 24, 48 or 72 hours of each other. In preferred embodiments, they are administered within 6, preferably within 2, more preferably within 1, most preferably within 20 minutes of each other. ***Please advise on preferred ranges***

In a preferred embodiment, they are administered as a pharmaceutical composition, which will generally comprise a suitable pharmaceutical excipient, diluent or carrier selected dependent on the intended route of administration.

Binding members and chemotherapeutic agents of and for use in the present invention may be administered to a patient in need of treatment via any suitable route. The precise dose will depend upon a number of factors, including the precise nature of the member (e.g. whole antibody, fragment or diabody) and chemotherapeutic agent.

Some suitable routes of administration include (but are not limited to) oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. Intravenous administration is preferred.

It is envisaged that injections (intravenous) will be the primary route for therapeutic administration of compositions although delivery through a catheter or other surgical tubing is also envisaged. Liquid formulations may be utilised after reconstitution from powder formulations.

For intravenous, injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives,

stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

The binding member, agent, product or composition may also be administered via microspheres, liposomes, other microparticulate delivery systems or sustained release formulations placed in certain tissues including blood. Suitable examples of sustained release carriers include semipermeable polymer matrices in the form of shared articles, e.g. suppositories or microcapsules. Implantable or microcapsular sustained release matrices include polylactides (US Patent No. 3, 773, 919; EP-A-0058481) copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al, Biopolymers 22(1): 547-556, 1985), poly (2-hydroxyethyl-methacrylate) or ethylene vinyl acetate (Langer et al, J. Biomed. Mater. Res. 15: 167-277, 1981, and Langer, Chem. Tech. 12:98-105, 1982). Liposomes containing the polypeptides are prepared by well-known methods: DE 3,218, 121A; Epstein et al, PNAS USA, 82: 3688-3692,

1985; Hwang et al, PNAS USA, 77: 4030-4034, 1980; EP-A-0052522; E-A-0036676; EP-A-0088046; EP-A-0143949; EP-A-0142541; JP-A-83-11808; US Patent Nos 4,485,045 and 4,544,545. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal rate of the polypeptide leakage.

Examples of the techniques and protocols mentioned above and other techniques and protocols which may be used in accordance with the invention can be found in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A. (ed), 1980.

The binding member, agent, product or composition may be administered in a localised manner to a tumour site or other desired site or may be delivered in a manner in which it targets tumour or other cells. Targeting therapies may be used to deliver the active agents more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons, for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

**Pharmaceutical Compositions**

As described above, the present invention extends to a pharmaceutical composition for the treatment of cancer, the composition comprising a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent and (c) a pharmaceutically acceptable excipient, diluent or carrier. Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention may comprise, in addition to active ingredients, a pharmaceutically acceptable excipient, carrier, buffer stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. intravenous.

The formulation may be a liquid, for example, a physiologic salt solution containing non-phosphate buffer at pH 6.8-7.6, or a lyophilised powder.

Dose

The binding members, agents, products or compositions are preferably administered to an individual in a "therapeutically effective amount", this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend

on the nature and severity of what is being treated. As described herein, the concentrations are preferably sufficient to show a synergistic effect. Prescription of treatment, e.g. decisions on dosage etc, is ultimately within the responsibility and at the discretion of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners.

The optimal dose can be determined by physicians based on a number of parameters including, for example, age, sex, weight, severity of the condition being treated, the active ingredient being administered and the route of administration. For example, with respect to binding members, in general, a serum concentration of polypeptides and antibodies that permits saturation of receptors is desirable. A concentration in excess of approximately 0.1nM is normally sufficient. For example, a dose of 100mg/$m^2$ of antibody provides a serum concentration of approximately 20nM for approximately eight days.

As a rough guideline, doses of antibodies may be given in amounts of 1ng/kg- 500mg/kg of patient weight. Equivalent doses of antibody fragments should be used at the same or more frequent intervals in order to maintain a serum level in

excess of the concentration that permits saturation of death receptor.

Doses of the binding members may be given at any suitable dose interval e.g. daily, once, twice or thrice weekly.

For example, the periods of administration of a humanised antibody could be from 1 bolus injection to weekly administration for up to one year in combination with chemotherapeutic agents. The likely dose is upwards of 1mg/per kg/per patient.

Doses of chemotherapeutic agent will depend on the factors described above but preferably are administered in doses which are within the normal range or, preferably, at a lower concentration than the normal range, wherein the normal range is the range of concentrations at which the chemotherapeutic agent is usually administered in the absence of other therapeutic agents.

It is anticipated that in embodiments of the invention the binding members and chemotherapeutic agent could be given in combination with other forms of chemotherapy or indeed radiotherapy.

Indeed it is believed that the advantages of the invention may also be obtained when using specific binding members of the invention and radiotherapy, even in the absence of chemotherapeutic agents.

Thus, in a tenth aspect of the invention, there is provided a method of killing cancer cells comprising administration of a therapeutically effective amount of a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) radoiotherapy treatment.

In a eleventh aspect, the present invention provides a method of treating cancer comprising administration of a therapeutically effective amount of a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) radiotherapy treatment to a mammal in need thereof.

The specific binding member and the radiotherapy may be administered simultaneously, sequentially or simultaneously. In preferred embodiments of the invention, the chemotherapeutic agent is administered prior to the radiotherapy.

The invention will now be described further in the following non-limiting examples. Reference is made to the accompanying drawings in which:

Figure 1A illustrates Northern blot analysis of Fas mRNA expression in MCF-7 cells 48 hours after treatment with no drug (C) or 5μM 5-FU. Equal loading was assessed by analysing β-tubulin mRNA expression.

Figure 1B illustrates Western blot analysis of Fas expression in MCF-7 cells 72 hours after treatment with no drug (C), 5μM 5-FU or 25nM RTX. Equal loading was assessed by analysing β-tubulin expression.

Figure 1C illustrates MTT cell viability assays in MCF-7 cells treated with no drug (control), CH-11 alone (250ng/ml), 5-FU alone (5μM), or co-treated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was highly synergistic (RI=2.40, $p<0.0005$).

Figure 1D illustrates MTT cell viability assays in MCF-7 cells treated with no drug (control), CH-11 alone (250ng/ml), RTX alone (25nM), or co-treated with RTX and CH-11. The decrease in cell viability for the combined treatment was highly synergistic (RI=2.22, $p<0.0005$).

Figure 1E illustrates analysis of apoptosis in 5-FU and CH-11 co-treated MCF-7 cells.

Figure 1F illustrates analysis of apoptosis in RTX and CH-11 co-treated MCF-7 cells. Apoptosis was assessed by analysing the sub-$G_1/G_0$ fraction of propidium iodide stained cells by flow cytometry. For both the MTT and flow cytometric analyses the cells were pre-treated with each chemotherapeutic drug for 72 hours followed by CH-11 for a further 24 hours.

Figure 2A illustrates Western blot analysis of Fas expression in HCT116p53$^{+/+}$ cells treated with a range of concentrations of 5-FU for 48 hours.

Figure 2B illustrates MTT cell viability assays in HCT116p53$^{+/+}$ cells treated with no drug (control), CH-11 alone (250ng/ml), 5-FU alone (5μM), or co-treated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was synergistic (RI=1.92, $p<0.005$).

Figure 2C illustrates Western blot analysis of Fas expression in HCT116p53$^{+/+}$ cells treated with a range of concentrations of RTX for 48 hours.

Figure 2D illustrates MTT cell viability assays in HCT116p53$^{+/+}$ cells treated with no drug (control), CH-11 alone (250ng/ml), RTX alone (50nM), or co-treated with RTX and CH-11. The decrease in cell viability for the combined treatment was highly synergistic (RI=3.44, $p<0.0005$).

Figure 2E illustrates Western blot analysis of Fas expression in RKO cells treated with a range of concentrations of 5-FU for 48 hours.

Figure 2F illustrates MTT cell viability assays in RKO cells treated with no drug (control), CH-11 alone (250ng/ml), 5-FU alone (5μM), or co-treated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was synergistic (RI=1.74, $p<0.005$).

Figure 2G illustrates Western blot analysis of Fas expression in RKO cells treated with a range of concentrations of RTX for 48 hours.

Figure 2H illustrates MTT cell viability assays in RKO cells treated with no drug (control), CH-11 alone (250ng/ml), RTX alone (5nM), or co-treated with RTX and CH-11. The decrease in cell viability for the combined treatment was highly synergistic (RI=2.31, $p<0.0005$). Equal loading of Western blots was assessed by analysing β-tubulin expression. For each combined treatment the cells were pre-treated with chemotherapeutic drug for 72 hours followed by CH-11 for a further 24 hours.

Figure 3A illustrates Western blot analysis of Fas, FasL, procaspase 8 and BID expression in MCF-7 cells treated with $IC_{50}$ doses of 5-FU (5μM) and RTX (25nM) for 72 hours. Equal loading was assessed using a β-tubulin antibody.

Figure 3B illustrates Western blot analysis of Fas, procaspase 8 and BID expression in MCF-7 cells treated no drug (control), CH-11 alone (250ng/ml), 5-FU alone (5μM) for 96 hours, or co-treated with 5-FU for 72 hours followed by CH-11 for a further 24 hours. Co-treatment with 5-FU and CH-11 resulted in activation of caspase 8 and BID as indicated by processing of procaspase 8 and full-length BID (lane 4).

Figure 3C illustrates Western blot analysis of procaspase 8 and PARP expression in HCT116p53$^{+/+}$ cells treated with no drug (control), 5μM 5-FU or 50nM RTX alone or in combination with 250ng/ml CH-11.

Figure 3D illustrates Western blot analysis examining the kinetics of caspase 8 activation and PARP cleavage in MCF-7 cells treated for 72 hours with 5μM 5-FU followed by 250ng/ml CH-11 for the indicated times.

Figure 3E illustrates Western blot analysing Fas, procaspase 8 and PARP expression in MCF-7 cells treated with 5μM 5-FU for 72 hours followed by 250ng/ml CH-11, 10μM IETD-fmk, or a combination of CH-11 and IETD-fmk for 24 hours.

Figure 4A illustrates tetracycline (tet)-regulated expression of a *TS trans*-gene in M7TS90 cells.

Figure 4B illustrates Western blot analysing the effect of TS induction (-tet lanes) on Fas up-regulation in M7TS90 cells in response to treatment with 10μM 5-FU, 100nM RTX or 1μM MTA for 72 hours.

Figure 4C illustrates an MTT assay showing the impact of TS induction (-tet) on viability of M7TS90 cells following treatment with 5-FU (10μM) or RTX

(100nM) in the presence of co-treatment with 250ng/ml CH-11.

Figure 4D illustrates the impact of TS induction on caspase 8 activation and processing of full-length (118kDa) PARP in M7TS90 cells treated with 5-FU (10μM), RTX (100nM) or MTA (1μM) followed by 250ng/ml CH-11.

Figure 4E illustrates Effect of exogenous TS expression on the induction of apoptosis in M7TS90 cells treated with 5-FU (10μM) RTX (100nM) or MTA (1μM) in the presence of co-treatment with 250ng/ml CH-11. Apoptosis was assessed by analysing the sub-$G_1/G_0$ fraction of propidium iodide stained cells by flow cytometry. Equal loading of Western blots was assessed by analysing β-tubulin expression. For each combined treatment the cells were pre-treated with chemotherapeutic drug for 72 hours followed by CH-11 for a further 24 hours.

Figure 5A illustrates Western blot analysis of Fas expression in p53 wild type (wt) M7TS90 and p53 null (nl) M7TS90-E6 cells 72 hours after treatment with no drug (Con), 10μM 5-FU, 100nM RTX or 1μM MTA.

Figure 5B illustrates MTT cell viability assays in p53 null M7TS90-E6 cells treated with 10μM 5-FU, 100nM RTX or 1μM MTA in combination with 250ng/ml CH-11.

Figure 5C illustrates Western blot analysis of procaspase 8 and full-length (118kDa) PARP expression in M7TS90 (wt) and M7TS90-E6 (nl) cells treated with 5-FU (10μM), RTX (100nM) or MTA (1μM) followed by 250ng/ml CH-11.

Figure 5D illustrates Effect of CH-11 (250ng/ml) on the induction of apoptosis in M7TS90-E6 cells treated with 5-FU (10μM) RTX (100nM) or MTA (1μM). Apoptosis was assessed by analysing the sub-$G_1/G_0$ fraction of propidium iodide stained cells by flow cytometry. Equal loading of Western blots was assessed by analysing β-tubulin expression. For each combined treatment the cells were pre-treated with chemotherapeutic drug for 72 hours followed by CH-11 for a further 24 hours.

Figure 6A illustrates Western blot analysis of Fas expression in HCT116p53$^{-/-}$ cells treated with a range of concentrations of 5-FU for 48 hours.

Figure 6B illustrates MTT cell viability assays in HCT116p53$^{-/-}$ cells treated with no drug (control), CH-11 alone (250ng/ml), 5-FU alone (10μM), or co-treated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was not synergistic (RI=1.01).

Figure 6C illustrates Western blot analysis of Fas expression in HCT116p53$^{-/-}$ cells treated with a range of concentrations of RTX for 48 hours.

Figure 6D illustrates MTT cell viability assays in HCT116p53$^{-/-}$ cells treated with no drug (control), CH-11 alone (250ng/ml), RTX alone (50nM), or co-treated with RTX and CH-11. The decrease in cell viability for the combined treatment was synergistic (RI=1.62, p=0.01).

Figure 6E illustrates Western blot analysis of Fas expression in H630 cells treated with a range of concentrations of 5-FU for 48 hours.

Figure 6F illustrates MTT cell viability assays in H630 cells treated with no drug (control), CH-11 alone (250ng/ml), 5-FU alone (10μM), or co-treated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was not synergistic (RI=0.99).

Figure 6G illustrates Western blot analysis of H630 cells treated with a range of concentrations of RTX for 48 hours.

Figure 6H illustrates MTT cell viability assays in H630 cells treated with no drug (control), CH-11 alone (250ng/ml), RTX alone (50nM), or co-treated with 5-FU and CH-11. The decrease in cell viability for the combined treatment was synergistic (RI=1.41, p<0.005). Equal loading of Western blots was assessed by analysing β-tubulin expression. For each combined treatment the cells were pre-treated with chemotherapeutic drug for 72 hours followed by CH-11 for a further 24 hours.

## MATERIALS AND METHODS

**Cell Culture.** All cells were maintained in 5% $CO_2$ at 37°C. MCF-7, H630 and RKO cells were maintained in DMEM with 10% dialyzed bovine calf serum supplemented with 1mM sodium pyruvate, 2mM L-glutamine and 50µg/ml penicillin/streptomycin (from Life Technologies Inc., Paisley, Scotland). M7TS90 cells (6) were maintained in 'MCF-7 medium' supplemented with 1µg/ml puromycin, 1µg/ml tetracycline (from Sigma, Poole, Dorset, England), and 100µg/ml G418 (from Life Technologies Inc). M7TS90-E6 cells (6) were maintained in 'M7TS90 medium' supplemented with 200µg/ml hygromycin (Life Technologies Inc). To induce expression of exogenous TS, cells were washed three times in 1xPBS and incubated in growth medium lacking tetracycline. HCT116 $p53^{+/+}$ and $p53^{-/-}$ isogenic human colon cancer cells were kindly provided by Professor Bert Vogelstein (John Hopkins University, Baltimore, MD). HCT116 cell lines were grown in McCoy's 5A medium (GIBCO) supplemented with 10% dialysed foetal calf serum, 50µg/ml penicillin-streptomycin, 2mM L-glutamine and 1mM sodium pyruvate.

**Northern blot analysis.** Northern blots were performed as described previously using a cDNA probe complementary to the *Fas* coding region (7). Equal loading was assessed using a β-tubulin cDNA probe.

**Western Blotting.** Western blots were performed as previously described (6). The Fas/CD95, Bcl-2 and BID (Santa Cruz Biotechnology, Santa Cruz, CA), caspase 8 (Oncogene Research Products, Darmstadt, Germany) and PARP (Pharmingen, BD Biosciences, Oxford, England) mouse monoclonal antibodies were used in conjunction with a horseradish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody (Amersham, Little Chalfont, Buckinghamshire, England). FasL rabbit polyclonal antibody (Santa Cruz Biotechnology) was used in conjunction with an HRP-conjugated donkey anti-rabbit secondary antibody (Amersham). TS sheep monoclonal primary antibody (Rockland, Gilbertsville, PA) was used in conjunction with an HRP-conjugated donkey anti-sheep secondary antibody (Serotech, Oxford, England). Equal loading was assessed using a β-tubulin mouse monoclonal primary antibody (Sigma).

**Cell Viability Assays.** Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay (12). Cells were seeded at 2,500 cells per well on 96-well plates 24 hours prior to drug treatment and then treated with a range of concentrations of 5-FU, RTX and MTA for 72 hours, following which time the agonistic Fas monoclonal antibody, CH-11 (MBL, Watertown, MA), was added (10-250ng/ml) for a further 24 hours. MTT (0.5mg/ml) was then added to each well and the cells incubated at 37°C for a further 3 hours. The culture medium was removed and formazan crystals reabsorbed in 200µL DMSO. Cell

viability was determined by reading the absorbance of each well at 570nm using a 96-well microplate reader (Molecular Devices, Wokingham, England).

**Flow Cytometric Analysis.** Cells were seeded at $1x10^5$ per well of a 6-well tissue culture plate. After 24 hours, 5-FU, RTX or MTA were added to the medium and the cells cultured for a further 72 hours, after which time 250ng/ml CH-11 was added for 24 hours. DNA content of harvested cells was evaluated after propidium iodide staining of cells using the EPICS XL Flow Cytometer (Coulter, Miami, Fl).

**Statistical Analyses.** The nature of the interaction between the chemotherapeutic drugs and CH-11 was determined by calculating the R index (RI), which was initially described by Kern and later modified by Romaneli (13, 14). The RI is calculated as the ratio of expected cell survival ($S_{exp}$, defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) to the observed cell survival ($S_{obs}$) for the combination of A and B ($RI=S_{exp}/S_{obs}$). Synergism is then defined as an RI of greater than unity. Romanelli *et al.* suggest that a synergistic interaction may be of pharmacological interest when RI values are around 2.0 (14). This method was selected because treatment with CH-11 alone had little effect on cell viability, which meant that other methods such as the median effect principle (15) and isobologram methods were not suitable (16). To further assess the statistical significance of the interactions,

the inventors designed a univariate ANOVA analysis using the SPSS software package. This was an additive model based on the null hypothesis that there was no interaction between the drugs.

RESULTS

**Fas is highly up-regulated in response to 5-FU and RTX.** Using DNA microarray profiling, the inventors previously identified the Fas death receptor as being highly up-regulated in response to 5-FU in MCF-7 cells (7). Northern blot analyses confirmed that Fas mRNA was up-regulated in MCF-7 cells 48 hours following treatment with an $IC_{60}$ dose (5μM) of 5-FU (Fig. 1A). Analysis of Fas protein expression in MCF-7 cells revealed that it was up-regulated by ~12-fold 72 hours after treatment with 5-FU (Fig. 1B). Fas was also highly up-regulated (by ~7-fold ) in response to treatment with an $IC_{60}$ dose (25nM) of RTX (Fig. 1B).

**The agonistic Fas monoclonal antibody CH-11 synergistically activates apoptosis in response to 5-FU and RTX.** To examine the role of the Fas signalling pathway in mediating the response of MCF-7 cells to 5-FU and RTX, the inventors used the agonistic Fas monoclonal antibody CH-11. Cells were treated with $IC_{60}$ doses of each drug for 72 hours, after which time they were treated with 250ng/ml CH-11 for a further 24 hours. Treatment with 5μM 5-FU alone resulted in a ~60% reduction in cell viability compared to control (Fig. 1C). Treatment with CH-11 alone without prior incubation with 5-FU caused a

modest ~6% decrease in cell viability. However, treatment with 5-FU followed by CH-11 was found to result in an ~84% decrease in cell viability. The combined treatment had an RI value of 2.40 indicating that the interaction was highly synergistic. This was further confirmed by ANOVA analysis, which indicated that the synergistic interaction between the drugs was highly statistically significant ($p<0.0005$). Similarly, treatment with 25nM RTX for 72 hours followed by CH-11 for 24 hours produced a highly synergistic decrease in cell viability (RI=2.22, $p<0.0005$, Fig. 1D). An IgM isotype control antibody had no effect on the cell viability of drug-treated cells (data not shown).

To assess the degree of apoptosis in MCF-7 cells treated with 5-FU and RTX individually, or in combination with CH-11, the inventors carried out flow cytometry of propidium iodide stained cells and analysed the sub-$G_1/G_0$ apoptotic fraction. Cells were treated with either 5-FU or RTX for 72 hours followed by 250ng/ml CH-11 treatment for 24 hours. The inventors found that CH-11 alone had little effect on apoptosis (Figs. 1E and F). Treatment with 5-FU alone for 96 hours resulted in a modest ~2-fold induction of apoptosis in response to 5μM 5-FU (Fig. 1E). However, addition of CH-11 to 5-FU-treated cells resulted in a dramatic increase in apoptosis, with a ~12-fold induction of apoptosis following co-treatment with 5μM 5-FU and CH-11. Similarly, the combination of RTX with CH-11 resulted in dramatic

activation of apoptosis, with ~60% of cells in the sub-$G_1/G_0$ apoptotic phase following combined treatment with 25nM RTX and CH-11 compared to ~11% in untreated control cells, ~16% in cells treated with RTX alone and ~18% in cells treated with CH-11 alone (Fig. 1F). The activation of apoptosis by CH-11 in 5-FU and RTX treated cultures was observed across a range of concentrations of each drug (Figs. 1E and F), indicating that the synergistic interaction between CH-11 and both drugs was due to activation of apoptosis.

The inventors next examined the ability of CH-11 to activate apoptosis in other cell lines. Treatment of HCT116p53$^{+/+}$ colon cancer cells with 5-FU resulted in potent up-regulation (>10-fold) of Fas expression after 48 hours (Fig. 2A). Furthermore, treatment with 5μM 5-FU followed by 250ng/ml CH-11 synergistically decreased cell viability in this line with an RI value of 1.92 ($p<0.005$). Similarly, RTX treatment dramatically increased Fas expression after 72 hours (Fig. 2C), while treatment with RTX followed by CH-11 resulted in a highly synergistic decrease in cell viability (Fig. 2D, RI=3.44, $p<0.0005$). The inventors also examined another p53 wild type colon cancer cell line, RKO. As was the case with both MCF-7 and HCT116p53$^{+/+}$ cells, both 5-FU and RTX treatment resulted in dramatic Fas up-regulation 48 hours post-treatment (Figs. 3E and F). Furthermore, treatment of RKO cells with 5-FU or RTX followed by CH-11 synergistically decreased cell viability with RI values of 1.74 ($p<0.0005$) and 2.31

(p<0.0005) respectively (Figs. 3F and G). These results indicate that CH-11 not only activates apoptosis of 5-FU- and RTX-treated MCF-7 breast cancer cells, but also of HCT116p53$^{+/+}$ and RKO colon cancer cells. The inventors also found that treatment with the antifolate MTA up-regulated Fas expression and synergistically interacted with CH-11 to decrease cell viability in all three cell lines (data not shown).

**Effect of 5-FU and RTX on Fas signal transduction.** The inventors next examined drug-induced activation of the Fas signalling pathway in response to 5-FU and RTX. Although Fas was highly up-regulated (>10-fold) in MCF-7 cells in response to $IC_{60}$ doses of either drug, FasL expression was unaffected (Fig. 3A). Surprisingly, neither caspase 8, nor its substrate BID were activated in 5-FU- or RTX-treated cells as indicated by a lack of down-regulation of the levels of procaspase 8 or full-length BID (Fig. 3A). The inventors subsequently analysed activation of the Fas pathway in MCF-7 cells following co-treatment with 5-FU and CH-11. Fas, procaspase 8 and BID expression levels were determined in cells treated with 5μM 5-FU for 72 hours followed by 250ng/ml CH-11 for 24 hours and compared to cells treated with 5-FU alone or CH-11 alone for the appropriate time periods (Fig. 3B). Treatment with CH-11 alone had no effect on Fas, procaspase 8 or BID expression (Fig. 3B, lane 2). As already noted, treatment with 5-FU alone resulted in dramatic up-regulation of Fas, but had no effect on procaspase 8

or BID expression, indicating that neither molecule was activated (Fig. 3B, lane 3). However, treatment of MCF-7 cells with 5-FU and CH-11 resulted in a dramatic activation of both caspase 8 and BID as indicated by complete loss of procaspase 8 and full-length BID expression in these cells (Fig. 3B, lane 4). Similarly, in HCT116p53$^{+/+}$ cells activation of caspase 8 was only observed following co-treatment with either 5-FU and CH-11 or RTX and CH-11 (Fig. 3C). Furthermore, cleavage of PARP (poly(ADP) ribose polymerase), a hallmark of apoptosis, was only observed in HCT116p53$^{+/+}$ cells co-treated with each drug and CH-11.

The inventors next compared the kinetics of caspase 8 activation with cleavage of PARP. Six hours after addition of CH-11 to MCF-7 cells pre-treated for 72 hours with 5μM 5-FU, procaspase 8 levels were reduced by ~3-fold compared to time zero (Fig. 3D). This coincided with PARP cleavage, which is indicative of cells undergoing apoptosis. Thus, activation of caspase 8 coincided with the onset of apoptosis. Twelve and 18 hours following CH-11 treatment, the levels of procaspase 8 had fallen to less than 5% of that observed at time zero, indicating potent activation of caspase 8. The inventors further examined the relationship between caspase 8 activation and apoptosis using the specific caspase 8 inhibitor IETD-fmk. Cells were pre-treated with 5μM 5-FU for 72 hours followed by 250ng/ml CH-11, 10μM IETD-fmk, or a combination of CH-11 and IETD-fmk for 24 hours. Fas was highly up-

regulated in all treatment groups (Fig. 3D). As noted above, the combination of 5-FU and CH-11 resulted in a dramatic activation of caspase 8 and PARP cleavage (Fig. 3E, lane 2). Addition of the caspase 8 inhibitor had no effect on protein expression in cells treated with 5-FU alone (Fig. 3E, lane 3). However, IETD-fmk blocked processing of procaspase 8 in cells co-treated with 5-FU and CH-11 (Fig. 3E, lane 4). This result indicates that caspase 8 activity is necessary for procaspase 8 processing at the DISC and is consistent with the induced proximity model proposed for caspase 8 activation (17). Significantly, blocking caspase 8 activation also inhibited PARP cleavage in 5-FU/CH-11 co-treated cells, indicating that apoptosis of these cells is dependent on caspase 8 activation.

**Effect of TS induction on the synergy between CH-11 and 5-FU, RTX and MTA.** Treatment with 5-FU and TS-targeted antifolates has been shown to acutely increase TS expression, most likely through disruption of a negative feedback mechanism in which TS binds to and inhibits translation of its own mRNA (18). This constitutes a potential mechanism of resistance as TS induction would facilitate recovery of enzymatic activity. The inventors therefore examined the effect of inducible TS expression on 5-FU and antifolate-mediated up-regulation of Fas and the synergistic interaction between CH-11 and each drug. To do this, the inventors used the MCF-7-derived M7TS90 cell line (6), in which transcription of a *TS trans*-gene is activated following withdrawal

of tetracycline (tet) from the culture medium (Fig. 4A). In agreement with the inventors' previous findings, TS induction in the M7TS90 cell line abrogated RTX- and MTA-, but not 5-FU-mediated up-regulation of Fas (Fig. 4B) (6). Furthermore, induction of the *TS trans*-gene had little effect on the synergistic interaction between 5-FU and CH-11 (Fig. 4C). However, TS induction completely abolished the synergistic decrease in cell viability caused by the combination of both 100nM RTX and CH-11 and 1μM MTA and CH-11 (Fig. 4C).

The inventors next assessed the effect of inducible TS on caspase 8 activation. The inventors found that TS induction abrogated caspase 8 activation in response to co-treatment with both antifolates and CH-11, but had no effect on caspase 8 activation in response to co-treatment with 5-FU and CH-11 (Fig. 4D). Similarly, TS induction abrogated processing of PARP in cells co-treated with the antifolates and CH-11, but not in cells co-treated with 5-FU and CH-11 (Fig. 4D). The differential effects of TS induction on apoptosis of 5-FU- and antifolate-treated M7TS90 cells was further analysed by flow cytometry by assessing of the sub-$G_0/G_1$ fraction in cells co-treated with drug and CH-11. Co-treatment with 5-FU and CH-11 resulted in a dramatic ~20-fold induction of apoptosis in M7TS90 cells that was only modestly reduced to ~17-fold when TS was induced (Fig. 4E). In contrast, RTX and CH-11 co-treatment resulted in a ~15-fold increase in the apoptoic fraction, which was reduced to ~5-fold by TS

induction (Fig. 4E). Similarly, combined treatment with MTA and CH-11 resulted in a dramatic ~26-fold induction of apoptosis that was almost completely abolished by inducible TS expression (Fig. 4E). These results indicate that the activation of Fas-mediated apoptosis in antifolate-treated cells was highly dependent on TS expression levels. In contrast, the 5-FU/CH-11 interaction was relatively insensitive to TS induction in this cell line, suggesting that non-TS-directed effects were primarily responsible for 5-FU cytotoxicity in these cells.

Effect of p53 inactivation on the synergy between CH-11 and 5-FU, RTX and MTA. The inventors next examined the role of p53 in the observed synergy between CH-11 and each drug. p53 has been reported to be an important regulator of Fas expression, both transcriptionally (19) and post-transcriptionally (20). The inventors previously described the generation of p53 null M7TS90-E6 cells by transfection of M7TS90 cells with human papilloma virus (HPV)-E6 (6). Treatment of these p53 null M7TS90-E6 cells with 10μM 5-FU, 100nM RTX or 1μM MTA did not result in Fas up-regulation (Fig. 5A). Furthermore, in contrast to the parental line, the combination of 5-FU and CH-11 did not synergistically decrease cell viability (RI=0.97, Fig. 5B). Similarly, inactivation of p53 also abolished the synergy between RTX and CH-11 and between MTA and CH-11 (RI=0.85 and 1.02 respectively, Fig. 5B).

The inventors further examined the effects of p53 inactivation on drug sensitivity by comparing caspase 8 activation in the p53 wild type and null isogenic M7TS90 lines. Activation of caspase 8 was not observed in the p53 null M7TS90-E6 cells co-treated with each drug and CH-11 (Fig. 5C). In contrast, caspase 8 was potently activated in the parental p53 wild type cell line in response to each co-treatment (Fig. 5C). Inactivation of p53 also completely attenuated PARP cleavage in response to co-treatment with 5-FU and CH-11 (Fig. 5C). However, processing of PARP was evident in p53 null cells

treated with both the RTX/CH-11 and MTA/CH-11 combinations, although to a lesser extent than in the p53 wild type line (Fig. 5C). As caspase 8 was not activated, this suggests that antifolate-mediated PARP cleavage in the p53 null cells was not due to activation of Fas-mediated apoptosis by CH-11. Indeed, the inventors found that PARP was also processed in the p53 null cell line in response to treatment with either RTX alone or MTA alone (data not shown). These results indicate that treatment with the antifolates activated p53-and Fas-independent apoptosis in M7TS90-E6 cells. This was further confirmed by flow cytometric analysis of apoptosis in the p53 null cell line. RTX (100nM) and MTA (1μM) significantly induced apoptosis of M7TS90-E6 cells by ~8-fold and ~6-fold respectively 96 hours after drug treatment (Fig. 5D). In contrast, little apoptosis was observed in M7TS90-E6 cells following treatment with 10μM 5-FU (Fig. 5D). Importantly, CH-11 had no significant effect on apoptosis induced by any of the drugs in the p53 null cell line.

The inventors extended their studies into the role of p53 in regulating antimetabolite-induced Fas-mediated apoptosis by examining the interaction between these drugs and CH-11 in the p53 null HCT116p53$^{-/-}$ cell line. This cell line was derived from the HCT116p53$^{+/+}$ cell line by somatic knock-out of both *p53* alleles (21). Compared to the p53 wild type cell line, there was very little Fas induction in response to 5-FU (Fig. 6A) and RTX (Fig. 6C) in

the HCT116p53$^{-/-}$ cell line, with an approximate 2-3-fold induction of Fas expression observed in response to 10μM 5-FU and 50nM RTX. Furthermore, no synergistic interaction was observed between 5-FU and CH-11 in the p53 null cell line (RI=1.01, Fig. 6B). Interestingly, a significant synergistic interaction was still observed between RTX and CH-11 in HCT116p53$^{-/-}$ cells (RI=1.62, p=0.01, Fig. 6D), although this was significantly less synergistic than the interaction observed in the p53 wild type parental line (Fig. 2D, RI=3.44, p<0.0005). This suggests that RTX-mediated sensitization of HCT116 cells to CH-11 is not wholly p53-dependent.

The role of p53 in mediating Fas-mediated apoptosis was further examined in the p53 mutant H630 colon cancer cell line. Similar to the p53 null cell lines, Fas was expression was not significantly altered in H630 cells in response to 5-FU (Fig. 6E) or RTX (Fig. 6G). No synergistic decrease in cell viability was observed between 5-FU and CH-11 (Fig. 6F, RI=0.99), however, a statistically significant synergistic interaction was observed between RTX and CH-11 (Fig. 6H, RI=1.64, p<0.0005). This interaction was observed despite the lack of any apparent up-regulation of Fas in response to this agent, suggesting that Fas expression is not the sole determinant of sensitivity to CH-11 in this cell line.

The inventors have observed similar synergistic interactions between anti-Fas monoclonal antibody and both TDX and oxaliplatin (data not shown) in

MCF-7 and HCT116 cell line models. Fas-targeted antibodies may thus be used to stimulate apoptosis in chemosensitised cancer cells.

DISCUSSION

The inventors have found that the Fas death receptor is highly up-regulated in response to 5-FU and the TS-targeted antifolates RTX and MTA in MCF-7 breast cancer and HCT116p53$^{+/+}$ and RKO colon cancer cells. However, this was in itself not sufficient to activate caspase 8. To mimic the effects of immune effector cells in their *in vitro* model, the inventors used the agonistic Fas monoclonal antibody CH-11. The inventors found that CH-11 potently activated Fas-mediated cell death in 5-FU- and antifolate-treated cells. Furthermore, the interaction between CH-11 and each drug was highly synergistic. The inventors' results suggest that the Fas signalling pathway is an important mediator not only of 5-FU-induced cell death, but also of antifolate-induced cell death.

The inventors found that although FasL was not induced following drug treatment, it was highly expressed in MCF-7 cells. Many tumour cells overexpress FasL, and it has been postulated that tumour FasL induces apoptosis of Fas-sensitive immune effector cells, thereby inhibiting the antitumor immune response. This hypothesis has been supported by both *in vitro* and *in vivo* studies (24, 25). The strategy of overexpressing FasL requires

that the tumour cells develop resistance to Fas-mediated apoptosis to prevent autocrine and paracrine induction of tumour cell death. Fas signalling may be inhibited by a Fas splice variant soluble Fas (sFas), which is a secreted protein that lacks the transmembrane domain of full-length Fas and may inhibit binding of FasL to Fas (26). Similarly, the Fas decoy receptor DcR3 is another secreted protein that binds to FasL with high affinity inhibiting its interaction with Fas (27). Downstream of Fas ligation, c-FLIP (FLICE-inhibitory protein) and FAP-1 (Fas-associated phosphatase-1) can inhibit caspase 8 recruitment and activation at the Fas DISC (28, 29). The lack of caspase 8 activation in response to treatment with 5-FU and the antifolates suggests that Fas-mediated apoptosis may be inhibited in MCF-7, HCT116 and RKO cancer cells. However, co-treatment with CH-11 was sufficient to overcome this resistance and activate Fas-mediated apoptosis.

The inventors' findings raise the possibility of using antimetabolite drugs in combination with anti-Fas antibodies as a novel anticancer strategy. Targeting Fas may be particularly useful against tumour cells that overexpress FasL and Fas pathway inhibitors, and which thereby evade Fas-mediated elimination by immune cells. However, systemic treatment with Fas antibodies or rFasL in mouse models has been shown to cause severe damage to liver and other organs (31). Some recent studies have focussed on local administration of rFasL, or

the use of FasL-expressing vectors as gene therapy to overcome systemic toxicity (31). In addition, a novel agonistic Fas-targeted antibody HFE7A has been developed recently that was not hepatotoxic in murine models, suggesting that it may be possible to develop less toxic Fas-targeted antibodies (32).

Treatment with TS inhibitors has been shown to acutely induce TS expression in cell lines and tumours (18, 33). Furthermore, pre-clinical and clinical studies have found that TS is a key determinant of sensitivity to 5-FU, with high TS expression correlating with increased resistance (1, 34). The inventors therefore examined the effect of elevated TS expression on activation of Fas-mediated apoptosis in 5-FU- and antifolate-treated cells using a tetracycline-regulated TS expression system (M7TS90). Interestingly, the inventors found that activation of apoptosis by CH-11 in response to 5-FU was not affected by increased TS expression. In contrast, TS induction completely abrogated the synergistic interaction between both RTX and CH-11 and MTA and CH-11. These findings correlated with Fas expression, the up-regulation of which was almost completely abrogated by TS induction in RTX- and MTA-treated cells, but not 5-FU-treated cells. These results indicate that the primary locus of 5-FU cytotoxicity in this cell line was not TS inhibition. Indeed, the inventors' previous studies have suggested that misincorporation of fluoronucleotides into RNA was the primary cytotoxic effect of 5-FU in this line (6). Thus, despite

expressing high levels of TS, certain tumours may still be sensitised to Fas-mediated apoptosis by 5-FU. However, high TS expression is likely to inhibit Fas-mediated apoptosis in response to folate-based TS inhibitors.

Several pre-clinical studies have demonstrated that loss of p53 function reduces cellular sensitivity to 5-FU (6, 21). Furthermore, a number of clinical studies have found that p53 mutations correlated with resistance to 5-FU, although other studies found no such association (34). The inventors assessed the effect of p53 inactivation on drug-induced Fas-mediated apoptosis in two p53 wild type and null isogenic cell line pairs: the MCF-7-derived M7TS90 and M7TS90-E6 lines, and the HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ lines. p53 inactivation attenuated Fas up-regulation in response to both drugs in both cell lines and inhibited the activation of apoptosis by CH-11 in 5-FU- and antifolate-treated cells; indicating that p53 is an important determinant of Fas-mediated apoptosis in response to these agents. Interestingly, some synergy was still observed between RTX and CH-11 in the HCT116p53$^{-/-}$ cell line, although it was significantly reduced compared to the p53 wild type cell line. The inventors also examined activation of Fas-mediated apoptosis in response to the antimetabolites in the p53 mutant H630 colon cancer cell line. Similar to the HCT116p53$^{-/-}$ cell line, little Fas induction was observed following drug treatment and no synergy was observed between 5-FU and CH-11. However, a

statistically significant synergistic interaction was again observed between RTX and CH-11. The inventors' results surprisingly suggest that RTX (but not 5-FU) can sensitize at least some cancer cell lines with non-functional p53 to Fas-mediated apoptosis. Furthermore, this effect appears to be independent of Fas up-regulation, suggesting that factors other than increased Fas expression contribute to the sensitisation of tumour cells to Fas-mediated apoptosis in response to this agent.

The inventors' data suggest that tumours with mutated p53 would be more resistant to Fas-mediated apoptosis in response to antimetabolites, in particular 5-FU. However, the discriminatory p53 mutants Pro-175 and Ala-143 have been shown to transcriptionally up-regulate Fas expression (35), suggesting that certain p53 mutant tumours may be sensitised to Fas-mediated cell death by chemotherapy.

In conclusion, the inventors have found that the agonistic Fas monoclonal antibody CH-11 dramatically increases the apoptotic response to 5-FU and TS-targeted antifolates in MCF-7, HCT116p53$^{+/+}$ and RKO cells. Induction of exogenous TS abrogated this synergistic interaction for the antifolates but not 5-FU, however, the extent of the interaction was highly p53-dependent for each drug. The inventors' findings suggest that the Fas signalling pathway is an important regulator of 5-FU- and antifolate-mediated cell death and that targeting the Fas

pathway in conjunction with either 5-FU or antifolates may have therapeutic potential.

The inventors have observed similar synergistic interactions between anti-Fas monoclonal antibody and both TDX (Fig.6) and oxaliplatin (data not shown) in MCF-7 and HCT116 cell line models. Fas-targeted antibodies may thus be used to stimulate apoptosis in chemosensitised cancer cells.

All documents referred to in this specification are herein incorporated by reference. Various modifications and variations to the described embodiments of the inventions will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes of carrying out the invention which are obvious to those skilled in the art are intended to be covered by the present invention.

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**Claims**

1. Use of (a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent in the preparation of a medicament for treating cancer.

2. The use according to claim 1 wherein the cancer is one or more of colorectal, breast , ovarian, cervical, gastric, lung, liver, skin and myeloid (e.g. bone marrow) cancer.

3. The use according to claim 1 or claim 2 wherein the binding member is an antibody or a fragment thereof.

4. The use according to any one of the preceding claims wherein the death receptor is FAS.

5. The use according to any one of the preceding claims wherein the binding member is the anti-FAS antibody CH11.

6. The use according to any one of the preceding claims wherein the binding member comprises at least one human constant region.

7. The use according to any one of the preceding claims wherein, wherein said active agent is 5-Fluorouracil or an antifolate.

8. The use according to claim 7 wherein said active agent is MTA.

9. A method of killing cancer cells comprising administering a therapeutically effective amount of a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent.

10. A method of treating cancer comprising administration of a therapeutically effective amount of a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent to a mammal in need thereof.

11. The method according to claim 9 or claim 10 wherein the cancer is one or more of colorectal, breast , ovarian, cervical, gastric, lung, liver, skin and myeloid (e.g. bone marrow) cancer.

12. The method according to claim 9, 10 or 11 wherein the binding member is an antibody or a fragment thereof.

13. The method according to any one of claims 9 to 12 wherein the death receptor is FAS.

14. The method according to any one of claims 9 to 13 wherein the binding member is the anti-FAS antibody CH11.

15. The method according to any one of claims 9 to 13 wherein the binding member comprises at least one human constant region.

16. The method according to any one of claims 9 to 15 wherein, wherein said active agent is 5-Fluorouracil or an antifolate.

17. The method according to claim 16 wherein said active agent is MTA.

18. A product comprising a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent as acombined preparation for the simultaneous, separate or sequential use in the treatment of cancer.

19. A pharmaceutical composition for the treatment of cancer, wherein the composition comprises a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent and (c) a pharmaceutically acceptable excipient, diluent or carrier.

20. The product according to claim 18 or the pharmaceutical composition according to claim

19 wherein the cancer is one or more of colorectal, breast , ovarian, cervical, gastric, lung, liver, skin and myeloid (e.g. bone marrow) cancer.

21. The product according to claim 18 or claim 20 or the pharmaceutical composition according to claim 19 or claim 20 wherein the binding member is an antibody or a fragment thereof.

22. The product according to claim 18 or claim 20 or 21 or the pharmaceutical composition according to claim 19 or claim 20 or 21 wherein the death receptor is FAS.

23. The product according to claim 18 or any one of claims 20 to 22 or the pharmaceutical composition according to claim 19 or or any one of claims 20 to 22 wherein the binding member is the anti-FAS antibody CH11.

24. The product according to claim 18 or any one of claims 20 to 23 or the pharmaceutical composition according to claim 19 or or any one of claims 20 to 23 wherein the binding member comprises at least one human constant region.

25. The product according to claim 18 or any one of claims 20 to 24 or the pharmaceutical composition according to claim 19 or or any one of claims 20 to 24 wherein, wherein said active

agent is 5-Fluorouracil or an antifolate.

26. The product or pharmaceutical composition according to claim 25 wherein said active agent is MTA.

27. 27. A kit for the treatment of cancer, said kit comprising:
a) a specific binding member which binds to a cell death receptor or a nucleic acid encoding said binding member and (b) a chemotherapeutic agent and
(c) instructions for the administration of (a) and (b) separately, sequentially or simultaneously.

Figure 1A

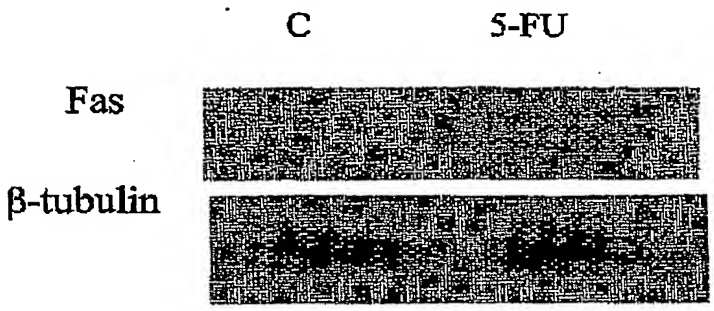



Figure 1B

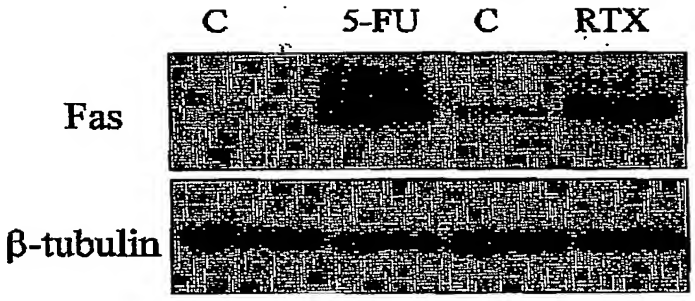


Figure 1C

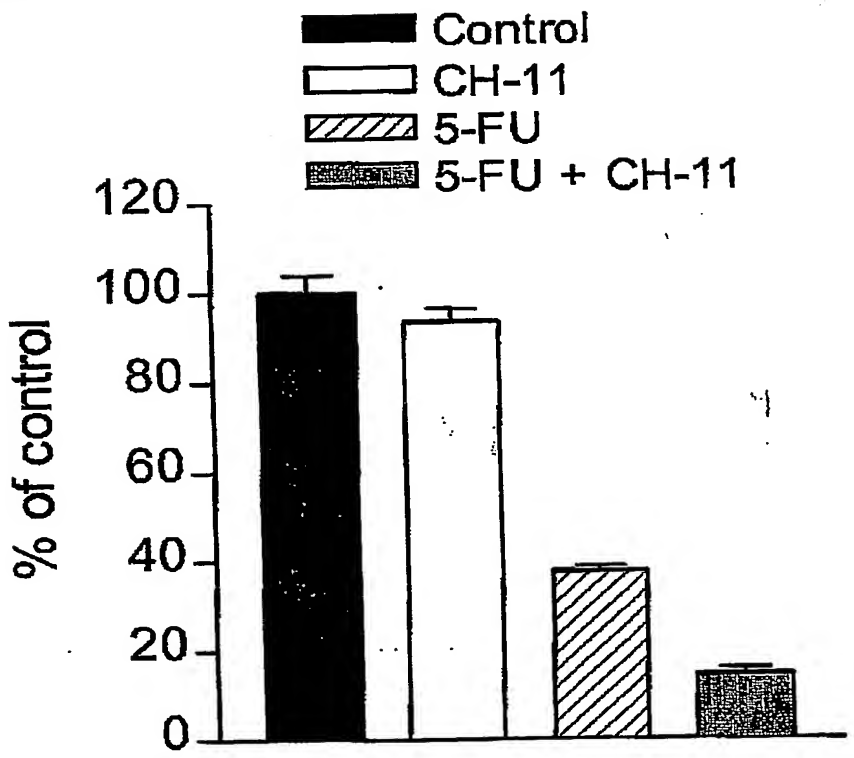
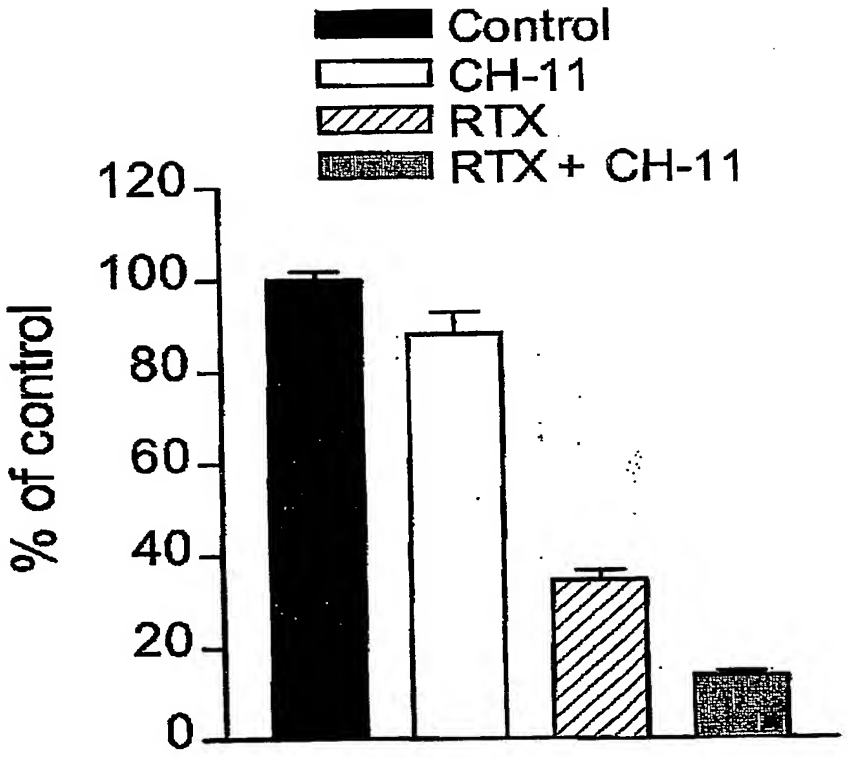


Figure 1D



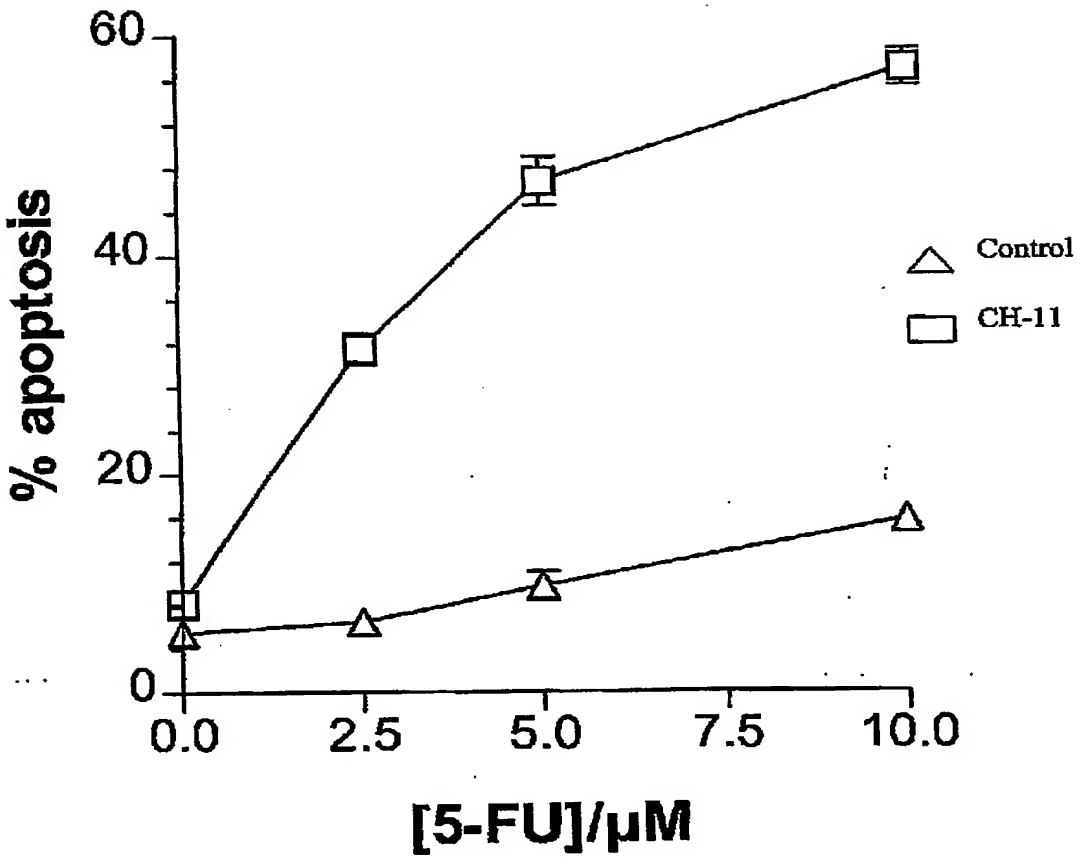



Figure 1E

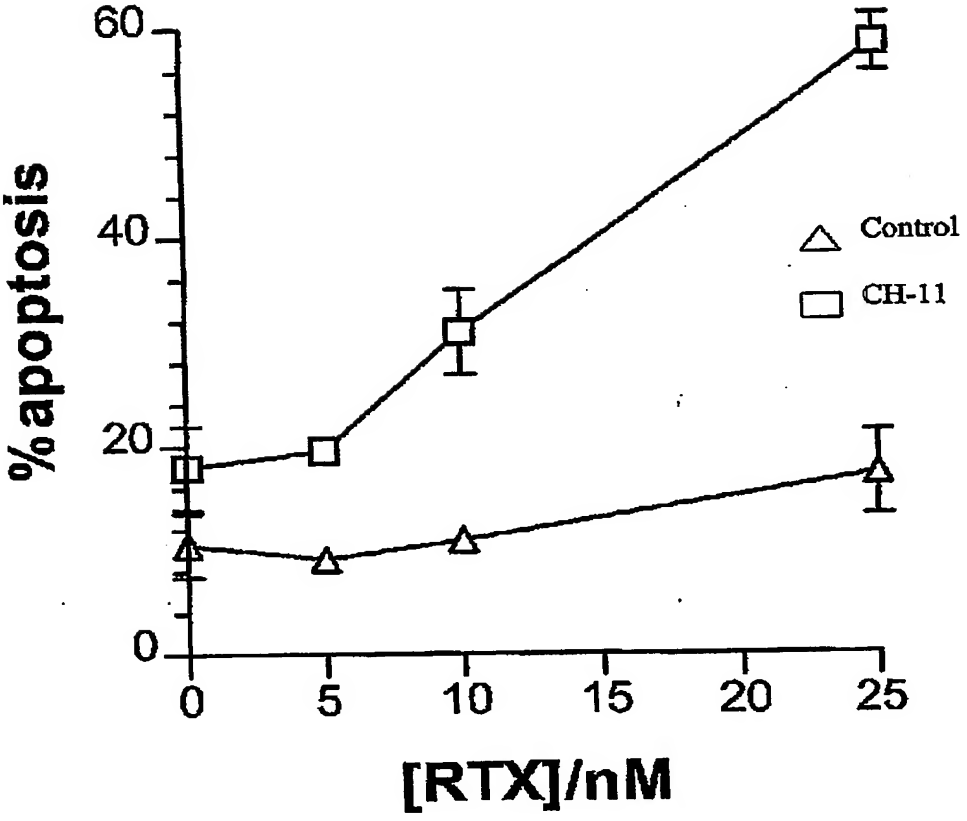
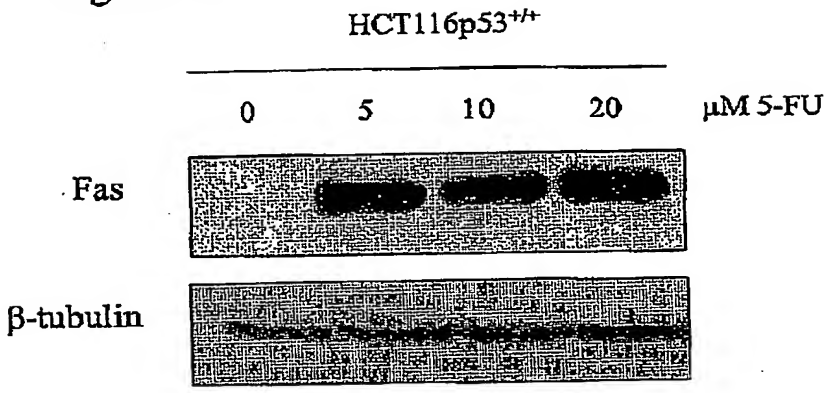



Figure 1F

Figure 2A



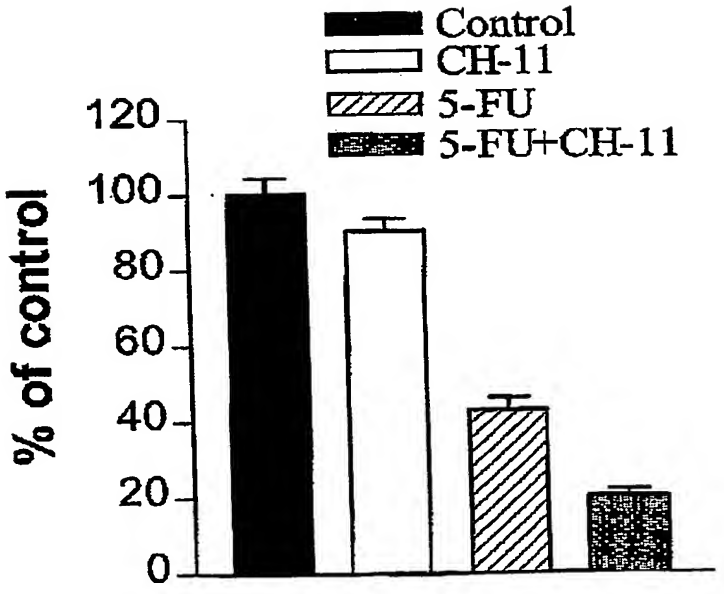




Figure 2B

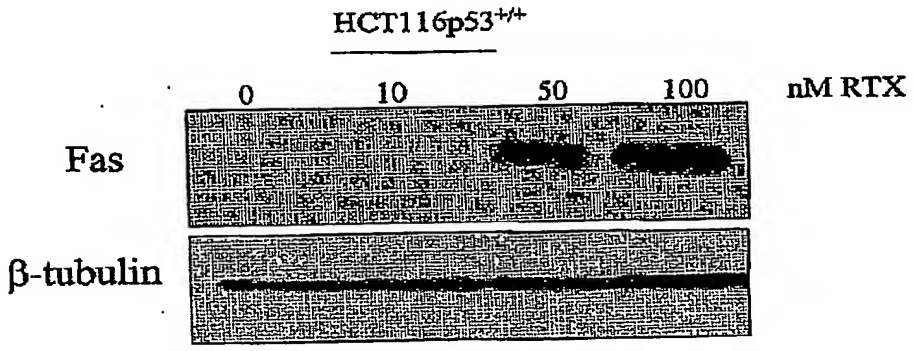



Figure 2C

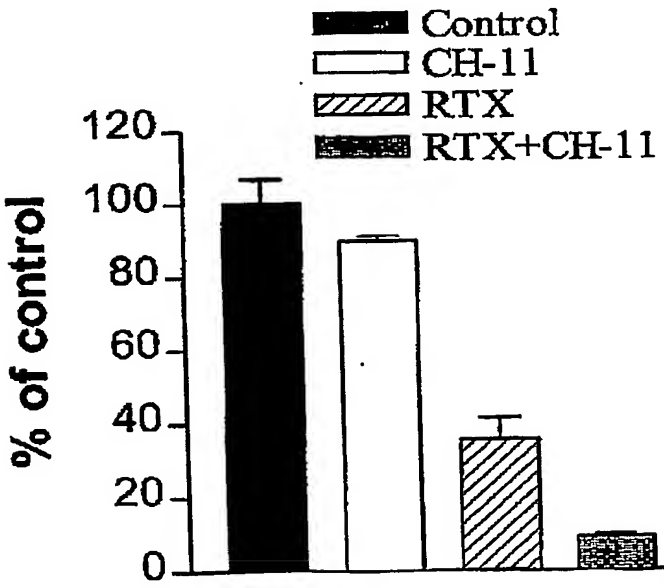



Figure 2D

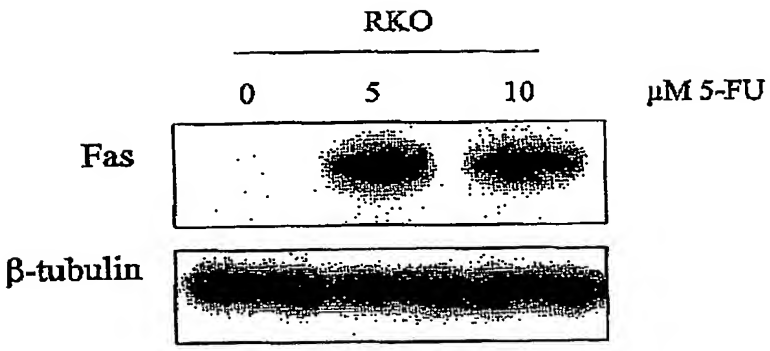



Figure 2E

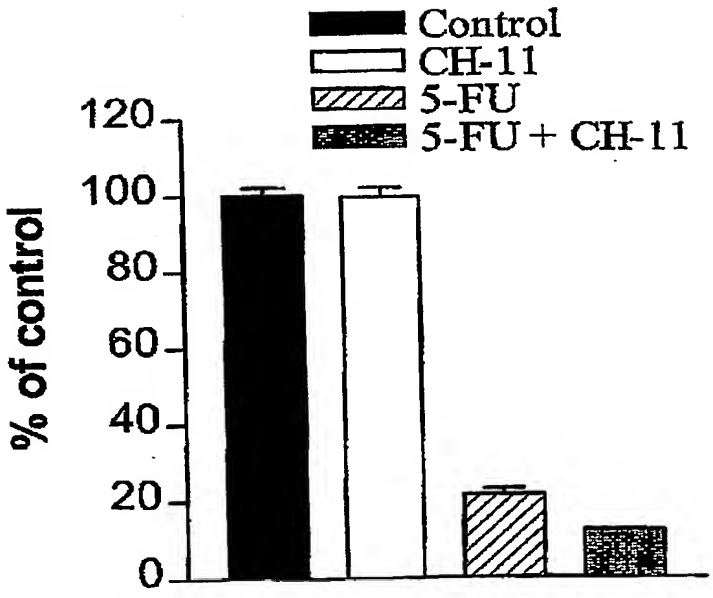



Figure 2F

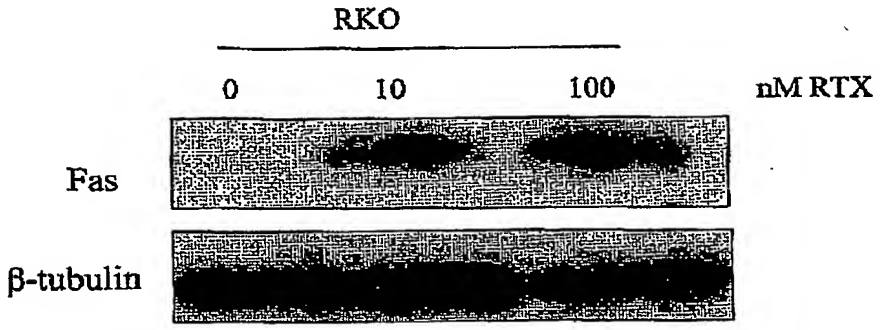



Figure 2G

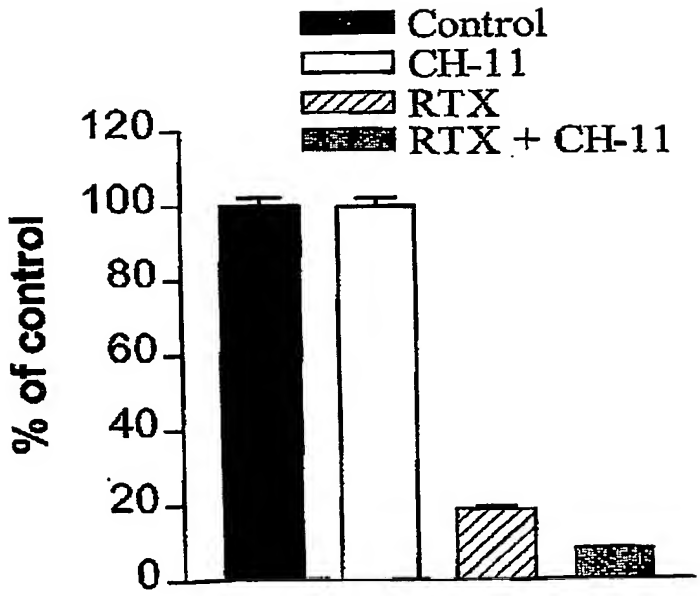
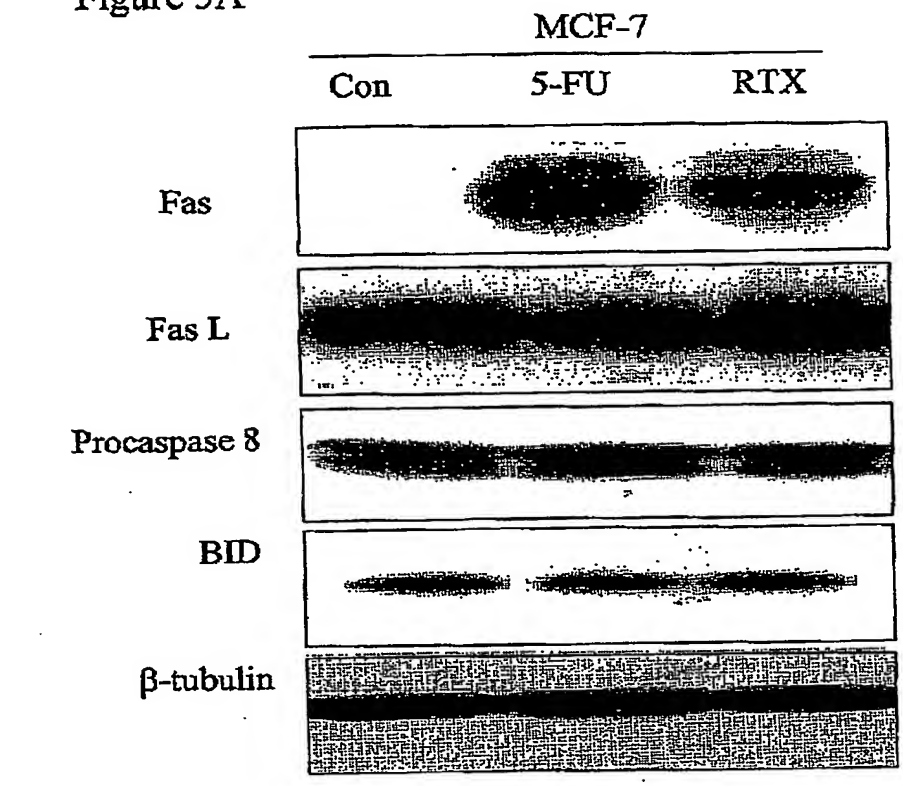



Figure 2H

Figure 3A



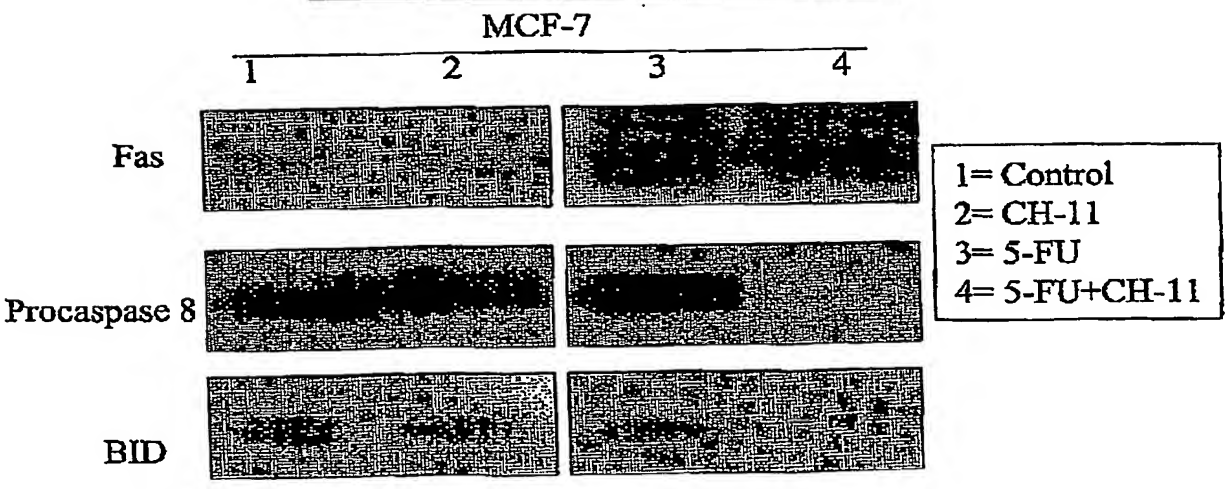




Figure 3B

Figure 3C

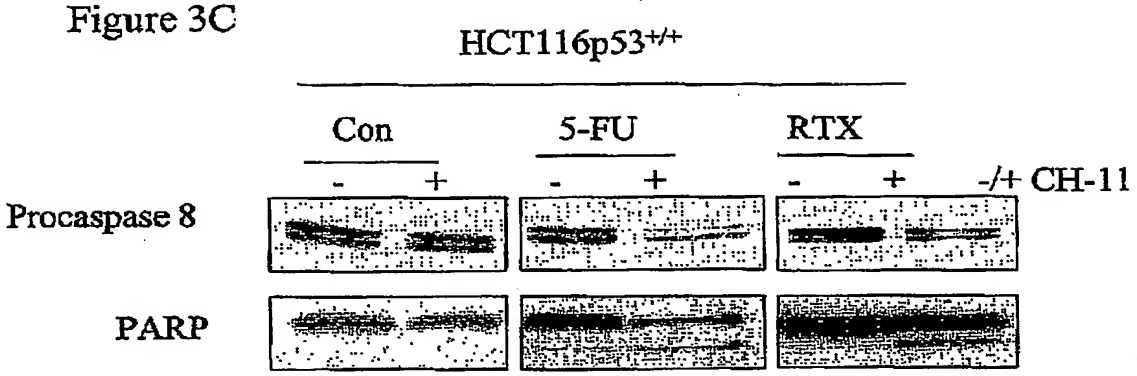



Figure 3D

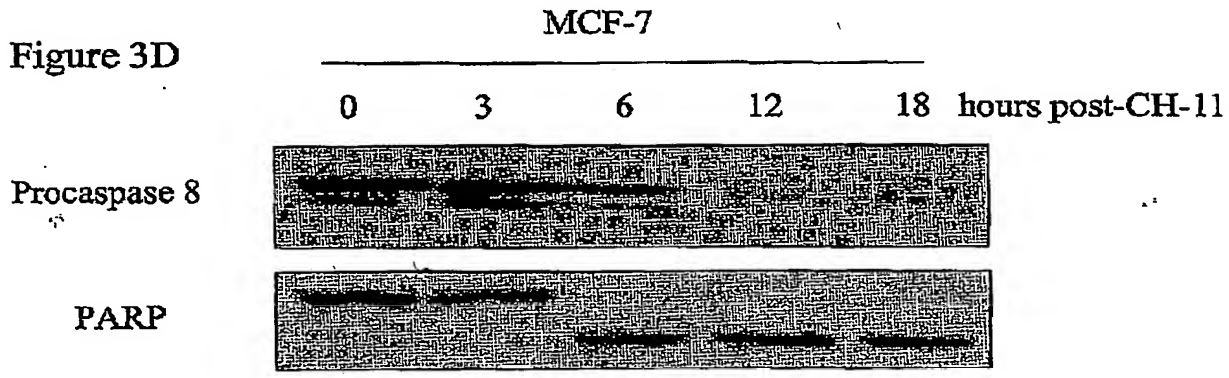



Figure 3E

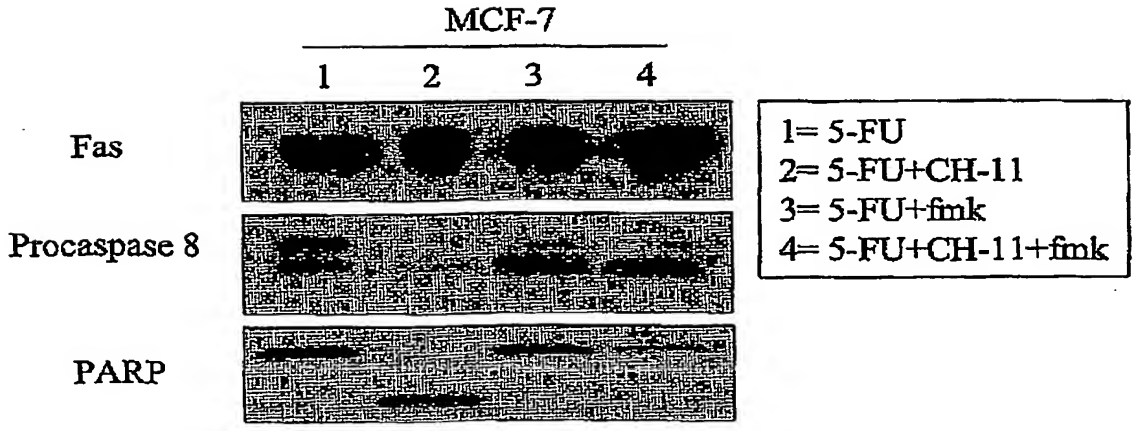


Figure 4A

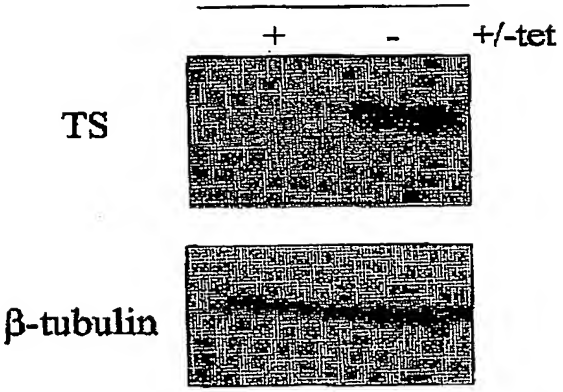



Figure 4B

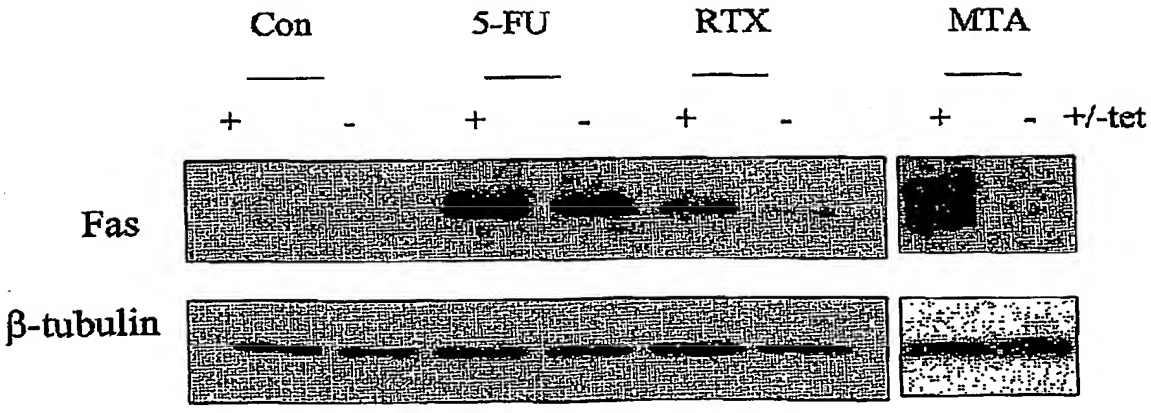


Figure 4C

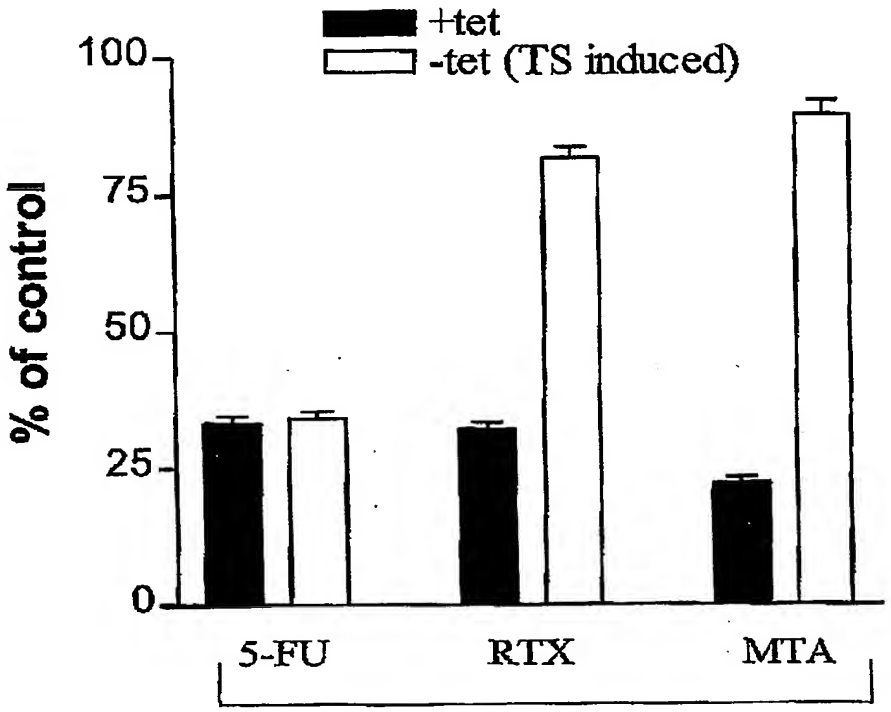


Figure 4D

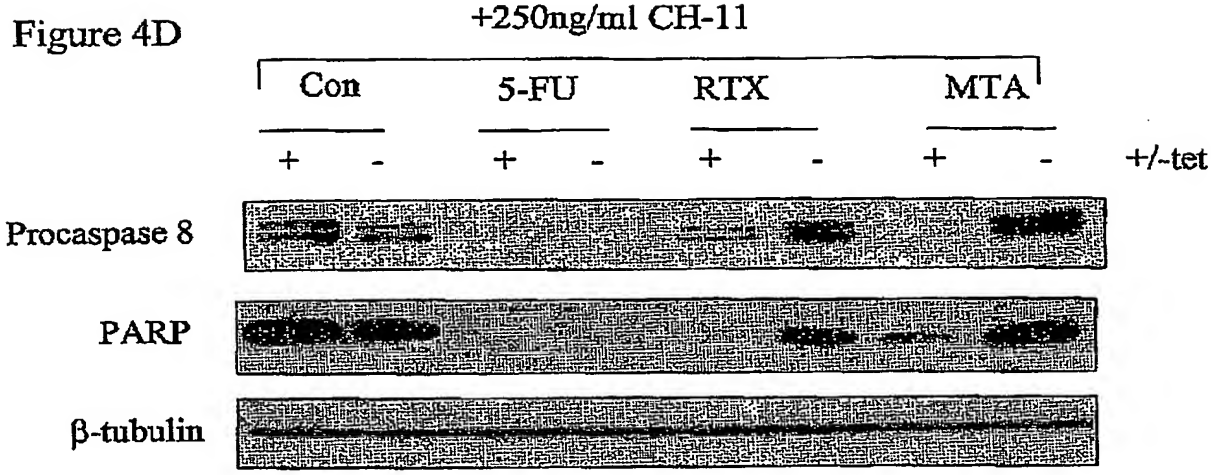



Figure 4E

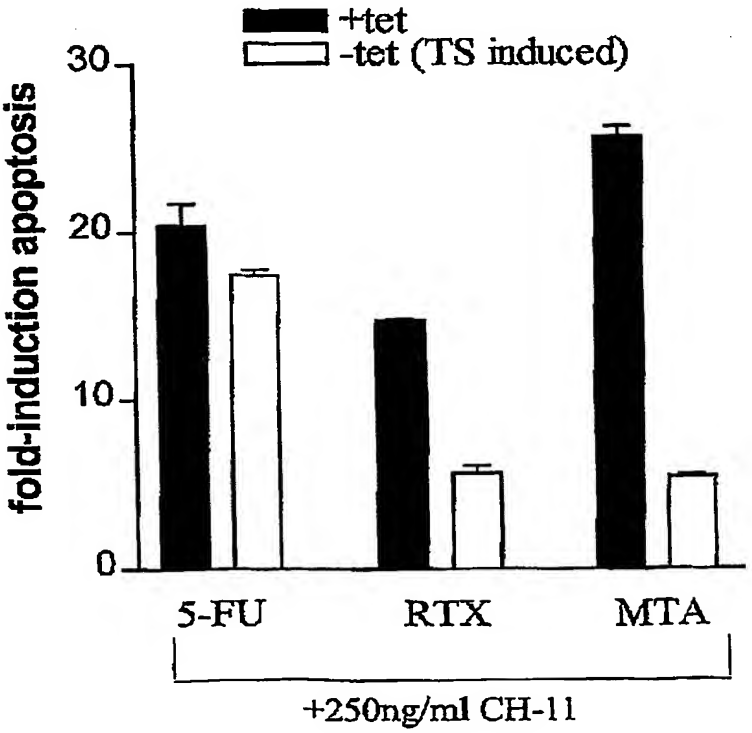


Figure 5A

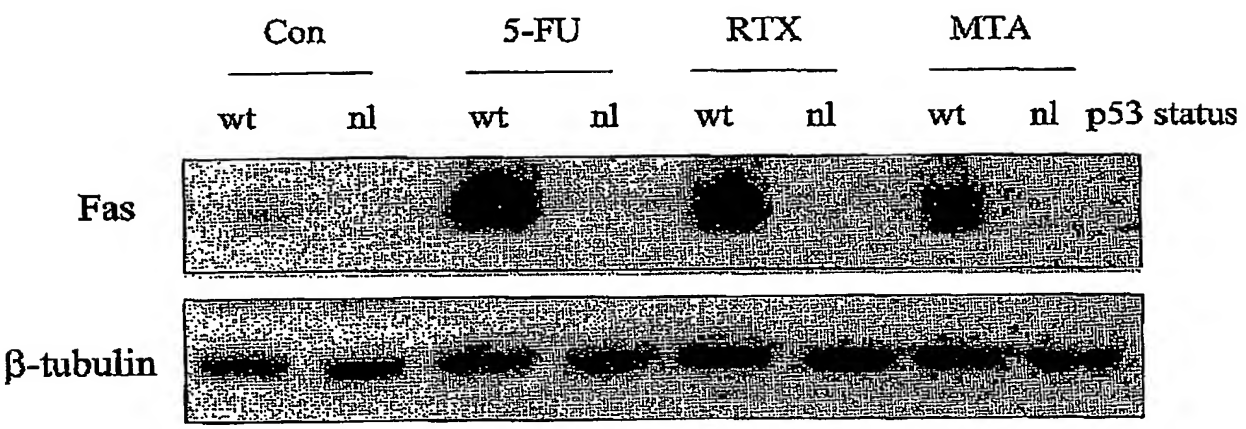



Figure 5B

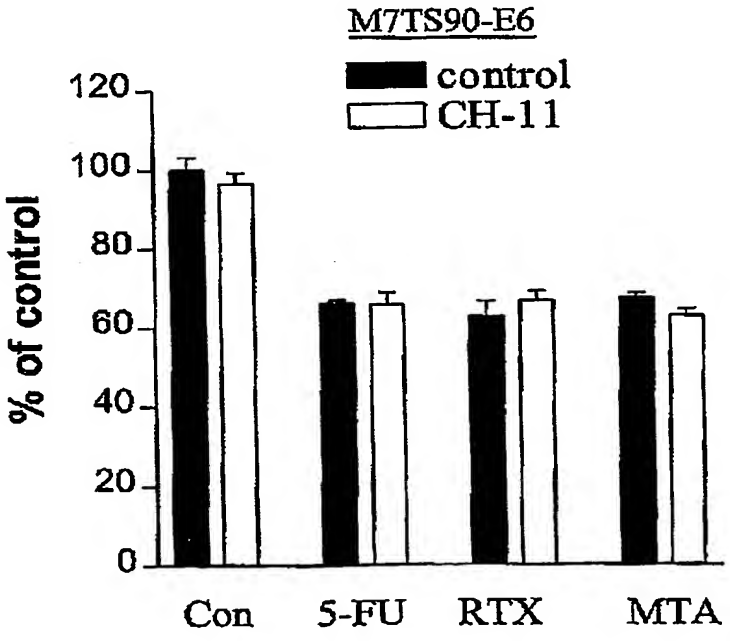


Figure 5C

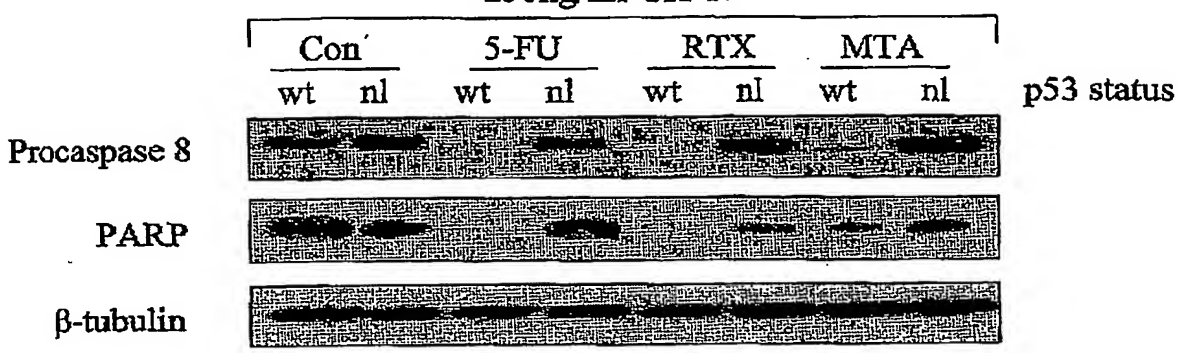



Figure 5D

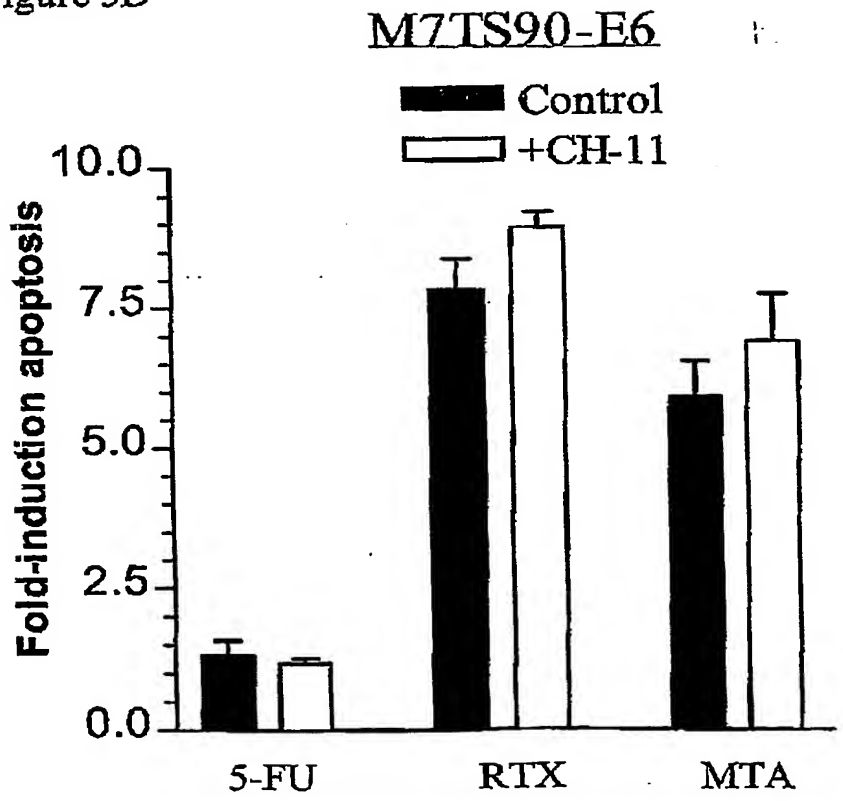


Figure 6A

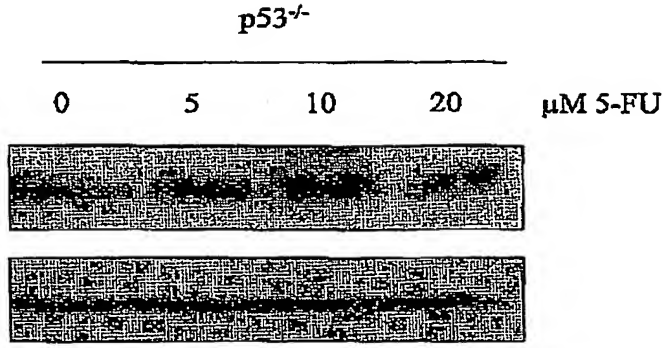



Figure 6B

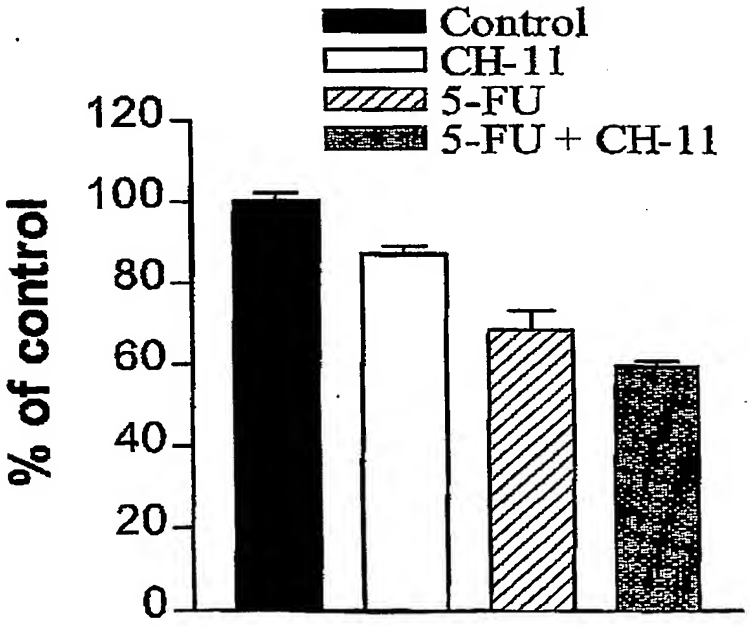


Figure 6C

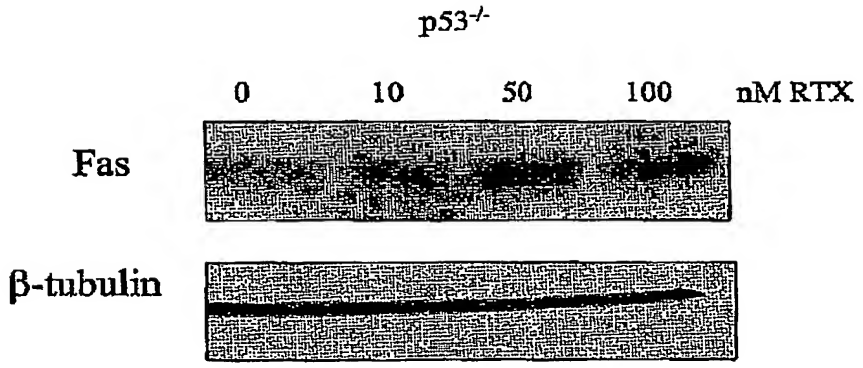



Figure 6D

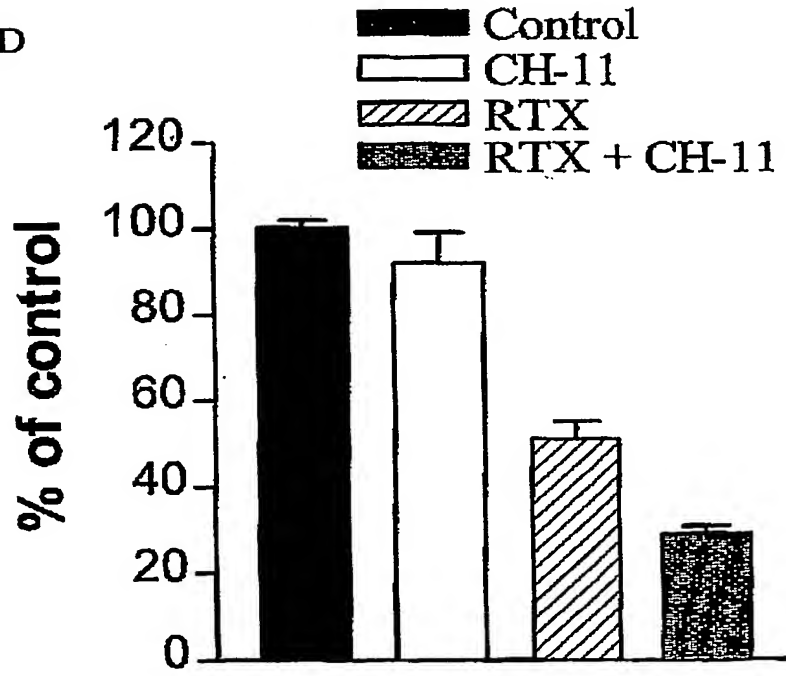


Figure 6E

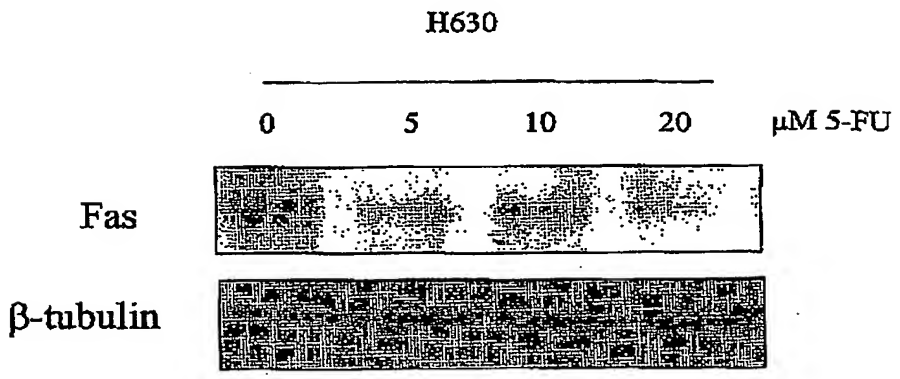



Figure 6F

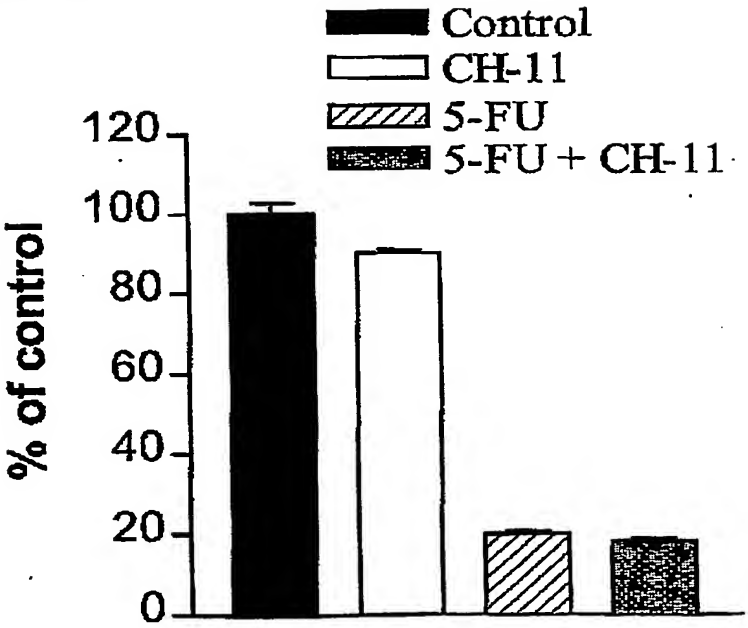


Figure 6G

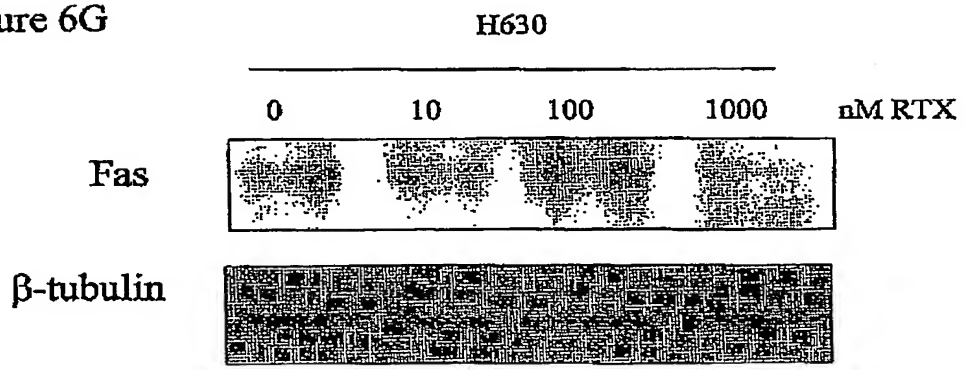



Figure 6H